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A Study of the Permeability and Transport Properties of Reconstituted Cell Membranes

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FOREWORD

This is one of a continuing series of reports designed to present accounts of progress in saline water conversion and the economics of its application. Such data are expected to contribute to the long-range development of economical processes applicable to low-cost demineralization of sea and other saline water.

Except for minor editing, the data herein are as contained in a report submitted by the contractor. The data and conclusions given in the report are essentially those of the contractor and are not necessarily endorsed by the Department of the Interior.

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SUMMARY

A program of exploratory research was undertaken to determine the permeability properties of natural lipoprotein films derived from red cell membranes. It was anticipated that the results of this study would provide information to permit the design and fabrication of synthetic membranes with properties approaching those of the natural systems. This work was divided into two major efforts, which were pursued concurrently: (1) A study of the physical chemistry, especially the preparation variables, of the lipoprotein membranes, and (2) Measurement of the permeability properties of these membranes on porous supports.

Earlier studies in our laboratory had demonstrated that thin lipoprotein membranes having many characteristics of the intact red cell membrane could be prepared by reconstituting a soluble lipoprotein preparation of the membrane at an air-water interface in the presence of a metal salt. In order to optimize the strength of these films, the surface chemistry of their components has been examined in detail and a brief study of membrane protein conformation was made using circular dichroism.

The surface pressure-area isotherms were determined as a function of subphase cation, anion, ionic strength and pH. A curve with two linear portions intersecting at the collapse pressure (π_c) was generally found. At pH 7.4 and constant ionic strength, π_c ranged from 18 to 22 dynes/cm for the chlorides of Na^+, K^+, Li^+, Mg^{++} and Ca^{++}. Narrow ranges were also observed for the collapse areas (3-8Å^2/residue) and film thicknesses (63-156Å). The compressibility modulus, $C_s^{-1} = -A \left(\frac{d\pi}{dA}\right)_{\pi = \pi_c}$, falling between 8 and 16 dynes/cm, was typical of values found for protein films. $LaCl_3$ produced the strongest and thickest film (π_c = 32 dynes/cm), but only at lower pH (4.8). Changes in ionic strength had very little effect on π_c, except for the 1M NaCl subphase which weakened the film. $NiCl_2$ also gave a weak film. Subphase anion did influence π_c, giving film strengths for Ca salts that increased in the following order: $NO_3^- < Cl^- < SO_4^= < CH_3CO_2^-$. Variation of pH between 2 and 9 produced little effect over KCl subphases, but tended to increase π_c somewhat on either side of 7 with $CaCl_2$. Treatment of the preformed films with glutaraldehyde produced a marked increase in π_c.

The surface chemistry of the isolated membrane protein, model proteins and mixtures of these with extracted lipid was explored on 10mM $CaCl_2$. In general, added lipid had little effect on the π-A curves of the proteins. However, the pyridine extracted protein plus lipid curve was nearly identical to that of the soluble lipoprotein preparation, as was the curve for intact red cell ghosts.

Circular dichroism (CD) spectra of the membrane protein show that the secondary protein structure is retained during solubilization of the ghosts and extraction of the protein; but, whereas reconstitution of the soluble lipoprotein generates an identical CD spectrum to that of the intact ghosts, addition of extracted lipid to isolated protein does not. CD spectra of model

proteins plus extracted lipid were also obtained. There seems to be little relationship between the surface activity of proteins and their secondary structure. The gel electrophoresis patterns of soluble lipoprotein and isolated membrane protein are similar.

Attempts to measure the permeability of the unsupported lipoprotein film failed. Commercial porous support membranes were dip coated at 10 dynes/cm with the lipoprotein film by a modified Langmuir-Blodgett technique to produce up to 50 layers. Film uptake occurred on the upstroke only and was quantitative. Conductance measurements showed essentially no difference in the salt permeability of coated versus uncoated supports.

Two other methods of loading the support, soaking and filtration, were more successful. Direct osmosis tests on Millipore filters of three different pore sizes, 25Å, 250Å and 3000Å, with $MgSO_4$ gave salt flux decreases over the untreated support of up to 77%, 55% and 8%, respectively, and a water flux increase in the 25Å support of up to 28%. The use of glutaraldehyde and multiple treatments were most effective. Similar experiments with 100Å Sartorius filters produced maximum salt flux decreases of 97% and water flux increases of 2000%. Tests with other cellulosic and non-cellulosic supports were variable and inconclusive.

Reverse osmosis tests on Eastman cellulose acetate (CA) membranes impregnated with soluble lipoprotein or ghosts showed no effect on the RO-97 membrane; however, a small increase in salt rejection from 0 to 30% associated with a sharp reduction in water flux from 232 to 1.6 gfd occurred in the HT-00 membrane. Attempts to coat previously dried CA films with ghosts were not encouraging. CA films cast from formulations containing small quantities of cell membrane components displayed large changes in water flux without affecting the salt rejection. Increases in water flux were found with ghosts, soluble lipoprotein and isolated protein; a sharp decrease occurred with lipid alone. Similar experiments with cellulose acetate butyrate gave poor results.

The results of these studies reveal the dramatic effect imparted by natural membrane components on the permeability properties, especially the water flux, of synthetic membranes. Further development of these and similar systems could result in a new type of desalination membrane having improved water flux. Although direct measurement of the permeability properties of the unsupported lipoprotein film was not achieved, study of the physical chemistry of these films provided further insight into the structural organization of biological membranes.

I. INTRODUCTION

A. Background

Although a vast amount of research has been done on biological membranes over the years and large sums have also been spent in the development of a practical membrane for desalination by reverse osmosis, up to now very little conscious effort has been directed toward applying the results of the studies on natural membranes to the problem of desalination. This is surprising, on the one hand, since cell membrances possess quite remarkable permselectivity and other transport properties; but, on the other hand, formidable problems were encountered when one tried to carry out measurements on cell membranes that could be directly related to the permeability characteristics of synthetic polymer membranes. Some recent success in this direction has come with the study of the phospholipid bilayer membranes.[1,2] However, although of considerable intrinsic interest, these lipid membranes lack the fundamental permeability properties of natural membranes and much doubt has been expressed as to their suitability as models for natural membranes.[3] Since the latter are lipoprotein in nature, i.e., composed chiefly of phospholipid and protein, lipoprotein membranes might represent better model systems.

It is well known that the biological membrane possesses a number of unique permeability properties which are presumed to arise from its unusual composition and molecular structure. It is thus of value to investigate natural membranes and their components in order to elucidate the design principles that underlie their unusual functional properties. Although much research has been done on biological membranes, a detailed knowledge of their structure and their structure-function relationships remains a mystery.[4]

The mammalian red cell membrane has probably been studied more thoroughly than any other biological membrane. This is mainly because of its ease of isolation in pure form and in quantity.[5] An additional advantage of the red cell membrane is its functional (and presumably structural) simplicity as compared with nerve, muscle, mitochondrial and chloroplast membranes. It displays no excitability, photosynthesis, electron transport, or unusual dimensional changes. In spite of this, it possesses the same type of structural components as other cell membranes in similar proportions and many of the same enzyme systems, especially those involved in active transport.[5] Furthermore, all five processes of diffusion through biological membranes have been observed in the red cell. These diffusion mechanisms are:[6]

1. Simple diffusion - flow down the concentration gradient through the bulk matrix of the membrane (Fickian diffusion).

2. Facilitated diffusion - carrier assisted diffusion down the concentration gradient, the carrier usually residing in the membrane.

3. Filtration or bulk flow - passage of solute across the membrane via water-filled pores; dependent on relative permeability to water and solute.

4. Active transport - uphill transport of solute against its concentration gradient; requires metabolic energy.

5. Pinocytosis - the entry of external solutes via invagination of the plasma membrane and sealing off internally.

Of these processes, only (1) and (3) have been demonstrated in synthetic membranes; (2), (4) and (5) are currently restricted to biological membranes. However, there are no theoretical barriers to the operation of all these diffusion mechanisms in synthetic membranes; only the practical limitations of present day knowledge limit their exploitation.

The passive permeability of the red cell membrane to a large number of ions and molecules has been determined and the **reflection** coefficient, σ, and solute mobility, ω, have been measured in a few cases.[7] In Table I is a listing of the half time, $t_{1/2}$, for penetration of the human red cell by a variety of ions and molecules. It is generally assumed that both water and solutes permeate the red cell membrane via a combination of diffusion through the bulk phase of the membrane plus flow through water-filled pores. Using several methods, Solomon and co-workers [8,9] have found the equivalent pore radius of the human red cell membrane to be about 4 Å. This would explain the low cation permeability of the membrane and the relatively greater permeability to K^+ (solvated radius = 2.54 Å) than to Na^+ (solvated radius = 3.78 Å). In contrast, the anion permeability is relatively high, a result which has been interpreted by Passow as evidence for the presence of positive fixed charges in the membrane, presumably the protonated amino groups of lysine units in the protein.[10]

A number of substances, especially sugars, are transported across the red cell membrane via carrier-facilitated diffusion. These carrier systems or permeases have been intensively studied by Wilbrandt,[11] LeFevre[12] and Stein[13] and are usually found to have high structural specificity for the molecule transported. Both allosteric interactions and chemical coupling have been proposed for these processes, but a unified theory of permease action is lacking.

The role of pinocytosis is well established in the digestive process and in the clearing of foreign particles from the circulation by lymphocytes and the reticuloendothelial system. Also, mammalian red cell membranes have recently been observed to undergo pinocytotic invagination when energized with ATP (adenosinetriphosphate).[14]

"Active transport" by the red cell membrane has been the subject of intensive study over the last decade. Many aspects of the uphill pumping

TABLE I

Permeability of Red Cell to Various Substances

Substance	$t_{1/2}$ Penetration (sec.)
H_2O	0.003
methanol	0.13
Cl^-	0.2
HCO_3^-	0.2
ethanol	0.3
butanol	0.7
acetamide	0.9
ethylene glycol	1.7
$SO_4^=$	18
glycerol	60
oxalate	60
NH_4^+	180
maleate	420
malonamide	1,025
fumarate	4,400
erythritol	10,800
succinate	13,200
Na^+	72,000 (20 hours)
tartrate	130,000
K^+	156,000
Ca^{++}	100,000,000
sucrose	∞
choline	∞

of Na^+ and K^+ powered by ATP hydrolysis have been worked out,[15] but a detailed understanding of the process still eludes us. The Na,K-ATPase enzyme system, which drives the "pump", is bound to the membrane and survives the hemolysis of the intact cell to form ghosts and the further disruption of the ghosts. Although not yet tested, there is good reason to believe that some ATPase activity will still be present in reconstituted membranes. This would prove very useful in further studies of the "pump" since this membrane would represent a barrier across which the vectorial ion pumping could be conveniently studied.

In the last decade numerous models have been proposed for the biological membrane. Perhaps the most important group have been those based on the phospholipid bilayer leaflet structure originally advanced by Davson and Danielli[16] and revived recently by the preparation of bilayer leaflet membranes by Mueller et al.[1] These membranes have been shown to possess a number of the properties of natural membranes, including high water flux, ion permselectivity, resting potentials, and excitability. However, only passive, Fickian diffusion has been demonstrated with these membranes, and other properties, such as high electrical resistance and low stability, have differed markedly from those of the natural membrane. Since the biological membrane consists essentially of about equal parts of protein and lipid,[3] the essential protein component is lacking in these bilayer systems. A number of workers have attempted with varying success to add proteins to these lipid membranes.[17-26] It appears that the protein and phospholipids of the natural membrane are organized into tight lipoprotein complexes which are difficult to form in vitro.

In spite of the widespread knowledge of the lipid plus protein composition of biological membranes, until recently little effort had been devoted to the formation of reconstituted or synthetic model membranes from lipoproteins or lipid-protein mixtures. Interest in this area has been spurred by recent reports that the catalytic functions of certain delipidated enzymes reappear after recombination with lipids.[27,28] It was concluded that lipids act as structural co-factors of the lipoproteins. Some of the earliest studies on reconstituted lipoprotein membranes were carried out on detergent solubilized membranes of Mycoplasma and bacteria.[29-32] It was demonstrated by electron microscopy that intact membranes had been formed after removal of the detergent. More recently, Zahler and Weibel[33] have claimed success in reconstituting red cell membranes by recombining proteins and lipids derived from the red cell by solvent extraction.

Similar studies have been underway in our laboratory over the last several years. Initially, our plan was to break up the cell membrane mechanically into rather large pieces and then try to seal these pieces back together at an interface to form a large membrane. We subsequently discovered that this approach was not feasible, but that, when the cell membrane was further fragmented into lipoprotein subunits, these subunits readily reorganized interfacially into large membranes having a thickness (100 Å or less) and protein plus lipid composition nearly identical to that of the

intact cell membrane.[34] The general appearance of the reconstituted membrane in electron micrographs is also very similar to that of intact cells. Figure 1 is an electron micrograph[35] of a lipoprotein membrane reaggregated on a still water surface from ultrasonically solubilized human red cell ghost membranes. Note the typical railroad track pattern always displayed by intact cell membranes.[36]

B. Objectives

The principal objectives of this work were to investigate the permeability properties of lipoprotein films derived from the red cell membrane and the basic mechanisms which give rise to these properties. This type of study is important to the problem of desalination since it could provide the necessary information for the design and fabrication of synthetic membranes with permeability properties approaching those of the natural systems. The research program was divided into four parts, as follows:

1. Design and construction of equipment for measurement of membrane characteristics.

2. Study of the effect of preparation variables on the physical properties of the membranes.

3. Development of porous supports and techniques of layering membranes on supports.

4. Measurement of the permeability properties of the reconstituted membranes.

Essentially all of the proposed studies have been carried out in addition to several others which were devised during the course of this project. The results of these studies are contained in the following sections.

II. PREPARATIVE METHODS AND PROCEDURES

A. Red Cell Ghosts

Red blood cells can be treated by hypotonic hemolysis to remove the hemoglobin contents and leave pale white cell envelopes. These cell envelopes have been commonly referred to as "ghosts"; the membrane properties of the ghosts are similar to those of the intact red blood cell.[5] This system is ideal for the study of cell membranes because of its ease of preparation and its purity. In the present work the ghosts were prepared by a modification of the hemolysis procedure of Dodge, et al,[37] in which the phosphate buffer was replaced by tris and EDTA.[34] In this procedure the red cells were freed of plasma by washing twice in 1:10 dilutions with solution A (0.14 M NaCl, 0.001 M EDTA, 0.005 M tris, pH adjusted to 7.4 with HCl) and harvested after each wash by centrifugation at 4000 x g for 5 mintues. The cells were hemolyzed by a 1:20 dilution in solution B (0.014 M NaCl, 0.001 M EDTA, 0.005 M tris, pH 7.4) followed by two washes in solution C (0.014 M NaCl,

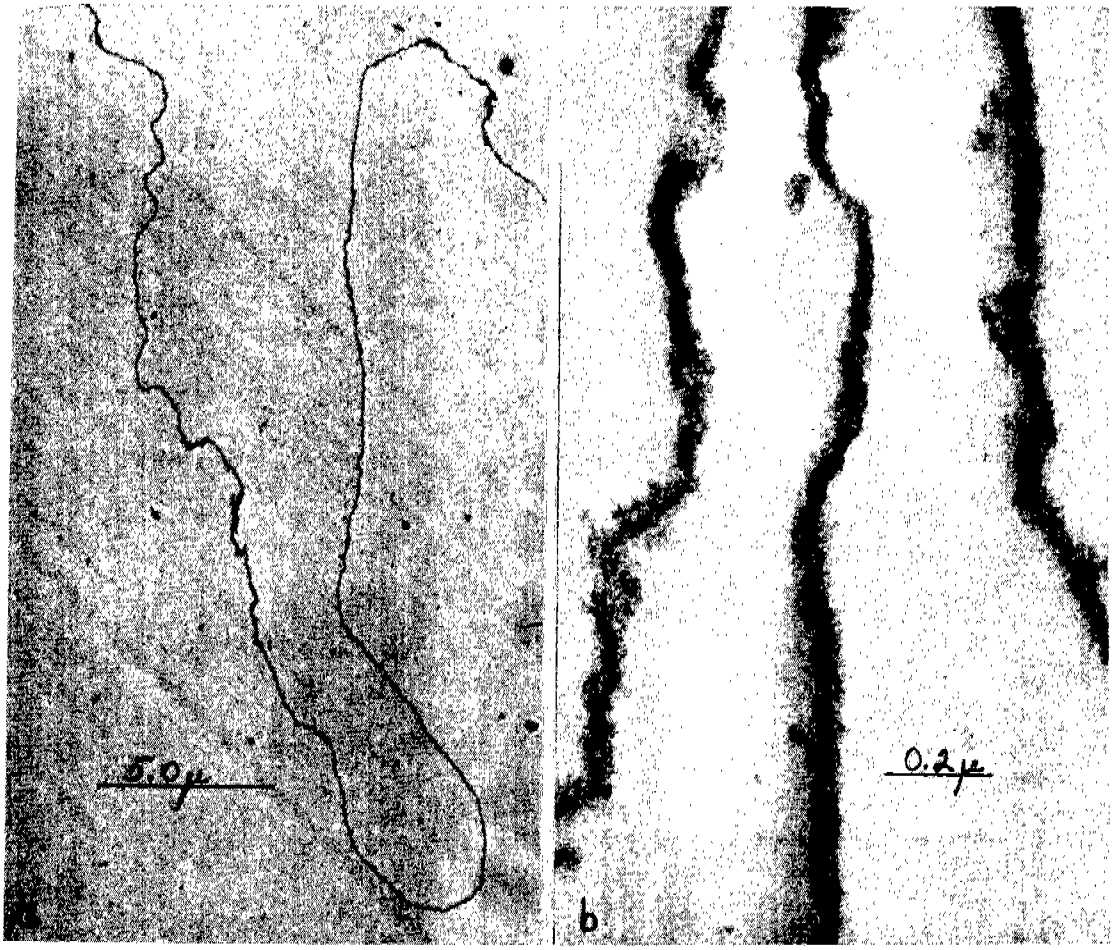

Figure 1 - (a) Electron micrograph of lipoprotein film reaggregated at an air-water interface from ultrasonically solubilized human red cell ghost membranes; (b) detail at higher magnification.

0.005 M tris, pH 7.4) After hemolysis and each wash, the ghosts were harvested by centrifugation at 14,000 x g for 10 minutes. The entire isolation procedure was carried out at 4°C.

B. Solubilized Ghost Membranes

It had been found previously in this laboratory that ghost cells can be broken up into much smaller units and reaggregated into very large ultrathin films in the presence of various cations.[34] The ghosts were disrupted by sonication (model S 75 Branson Sonifier at tap 8) in an ice bath. The membranes were disintegrated by successive 30 second bursts with cooling periods of about 3 minutes between each exposure until the solution had been sonicated for a total of 5 minutes. During this time the temperature of the solution was kept between 2° and 10°C.

The sonicated ghosts were separated into "soluble" and "insoluble" fractions by centrifugation at 90,000 x g for 2 hours at 4°C. The supernatant containing the lipoproteins which did not sediment will be referred to as the solubilized or sonicated preparation. All biological samples were stored at about 4°C and used promptly so as to avoid bacterial contamination.

C. Isolated Protein

The membrane protein was first isolated in these experiments by pyridine solubilization following a procedure developed by Blumenfeld.[38] In this procedure one-half volume of pyridine is added to one volume of ghost solution and the resulting mixture dialyzed overnight. The entire procedure is carried out at 4°C and about 40% of the total protein remains in solution after the dialysis step. The remainder of the protein and lipid precipitate are removed by centrifugation. The concentration of protein in solution was determined by the Folin[39] procedure. It was found that there was about 5-10% (based on protein) of phospholipid remaining in the final protein solution.

Another procedure for the isolation of membrane protein was also used. This was the butanol extraction technique developed by Maddy,[40] which was found to be considerably more complicated and less reproducible than the pyridine method. However, this method yields protein which has much less lipid still associated with it (less than 1% compared with 5-10% phospholipid for the pyridine procedure). In the Maddy procedure 10 ml of packed ghosts were centrifuged at 18,000 x g for 15 minutes and the resulting pellet frozen overnight at -20°C. The next day this preparation was washed three times with deionized-distilled water to remove any salt present. The ghost suspension was then cooled to 0°C in an ice bath, 7.5 ml of n-butanol at 0° was added, the tube shaken briefly to mix the contents, and held at 0°C for 15-20 minutes. The preparation was subsequently centrifuged for 4 minutes at 27,000 x g when it separated into a butanol phase, containing the lipid overlying the aqueous protein solution and a thin interfacial film

of insoluble protein. The lower phase was drawn into a cold hypodermic syringe, immediately transferred to a test-tube immersed in ice, and finally dialysed overnight in iced water.

D. Extracted Lipid

The lipid portion of the cell membrane was isolated by the chloroform - isopropanol extraction method of Rose and Oklander.[41] In this procedure 11 ml of isopropanol was added to 2 ml of ghost suspension and allowed to stand for one hour at room temperature. Seven ml of chloroform was then added. The resulting mixture was allowed to stand for another hour and finally centrifuged at 500 x g for 30 minutes. The lipid components remained in solution while the proteins were precipitated. Average yield of lipid was 20%. The phospholipid concentration was determined by the method of Chen and co-workers,[42] and it was shown that no detectable amounts of protein were present.

III. PHYSICAL CHEMICAL STUDIES

A. Surface Chemistry of Reconstituted Membranes

1. Apparatus

An automatic recording film balance using the Wilhelmy technique was constructed recently by the instrument group of the Research Service Department for use in surface chemical studies of high polymers. This instrument is uniquely suited to the studies on reconstituted membranes. Figures 2-4 show three views of the equipment. This balance has an automatic recording pressure sensing device for pressure-area curves and an automatic compression and expansion barrier for constant pressure experiments. Its moveable barrier is driven by an hydraulic system at either a constant rate with a synchronous motor or at a variable rate during constant pressure experiments with a Servo motor. The apparatus provides for constant temperature control and water level maintenance. The balance is equipped with a dipping device that is also activated by an hydraulic system.

The trough (with inside dimensions of 85 cm. x 15.2 cm. x 1.5 cm.) is made of aluminum with the inside coated with Teflon(R). The two long sides and tops are accurately machined. The long sides of the trough are fitted with guide rods upon which a carrier for the moveable barrier rides. The moveable barrier is a Teflon(R) coated bar which is fitted into slots on the carrier and is held in contact with the edges of the trough by springs.

The gear ratios of the synchronous motor and the compression rates of the moveable barrier are:

Gear Ratio	cm/hr
1:1	19.2
2:1	9.6
5:1	3.96
10:1	1.98
20:1	0.99
50:1	0.4

All runs reported in this study were made at a sweep rate of 19.2 cm/hr. A check of several systems at 3.96 cm/hr. gave the same π-A curve as the faster sweep rate.

The barrier is operated by a "slave unit" of an hydraulic system mounted under the trough. The slave unit is activated by a master hydraulic system consisting of oil lines, oil cylinder, filling and bleeding valves, and a mechanical drive. The mechanical drive consists of a threaded shaft, riding nut, synchronous motor and a servo unit. When the moveable barrier is operated at a constant rate, the synchronous motor is used. When operating at constant pressure, the Servo motor is used to drive the hydraulic system in either direction according to the signal and thus restore the pressure to the pre-set value.

The base of the trough is an aluminum heat exchanger with tubing for circulating water to maintain a constant temperature. The trough contains a well (80 mm x 90 mm x 5 mm) to accomodate the film supports during depositions of monolayers from the air-liquid interface to a solid support. The dipping device is mounted over the well in the trough and is moved at a constant speed by a slave unit of an hydraulic system for the dipping device. The trough and dipping device with the two slave units are mounted on a 3/4" thick aluminum base equipped with leveling screws. This whole unit is supported on a large table placed on Isomode pads to give a vibration free mounting. It is essential to have an environment free of vibrations, particularly those caused by vehicular traffic on nearby roads, motors, pumps, and large machinery. The master units of the two hydraulic systems are isolated from the trough on a separate laboratory bench. The controls for the moveable-barrier-hydraulic-system are located in the control cabinet.

The pressure sensing device uses a Wilhelmy plate and a recording Cahn RG Electrobalance. This balance has a "high-gain force-balance system" and only deflects 0.4 micron/mg. of force change. This change is so small that it is not necessary to correct for buoyancy effects. The balance is mounted above the

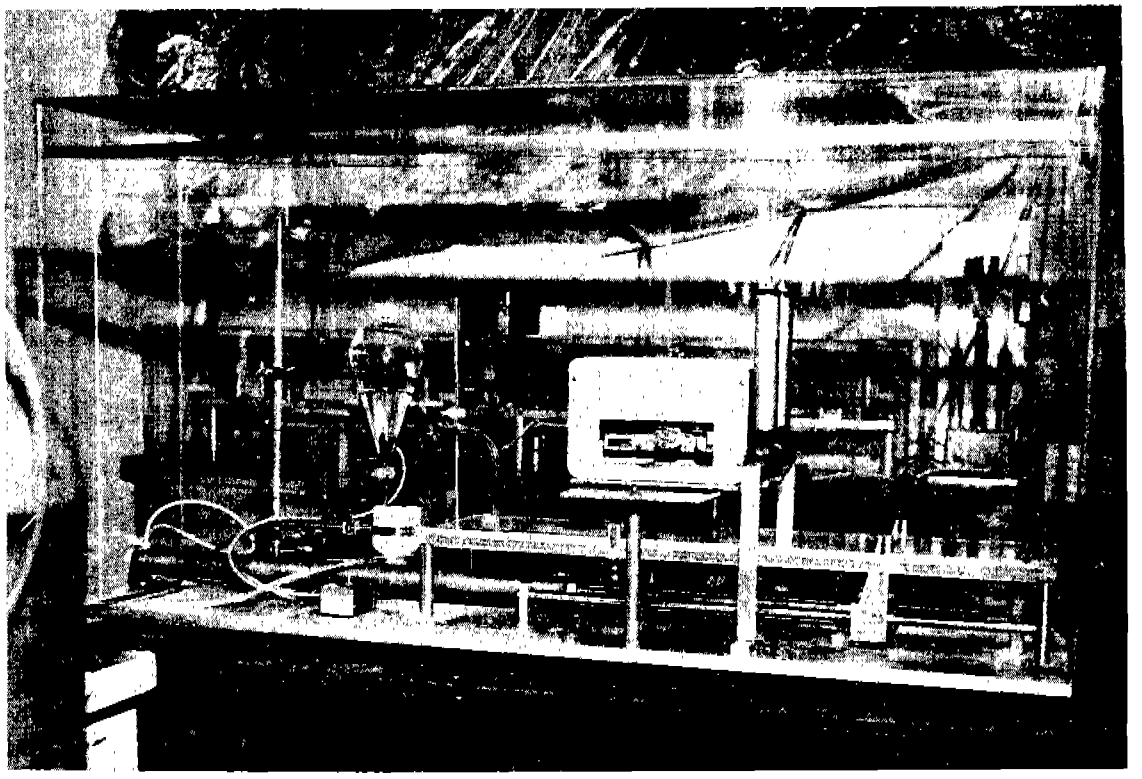

Figure 2 - General view of automatic recording film balance

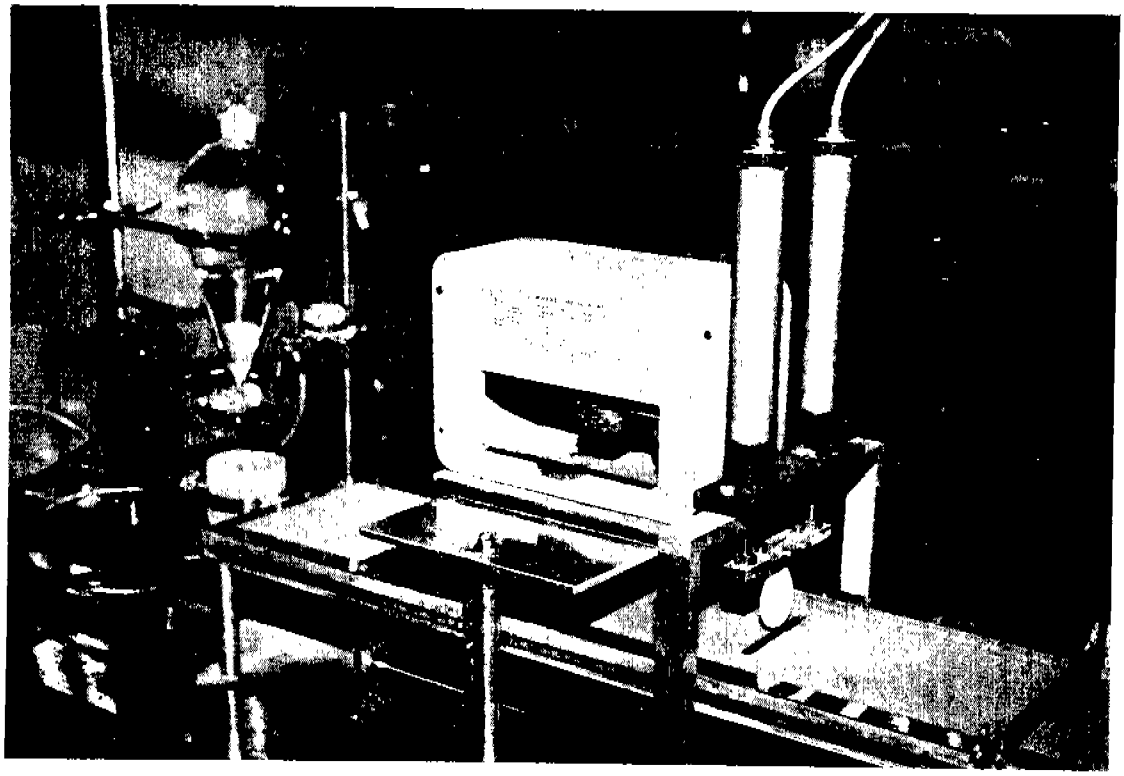

Figure 3 - Detail showing trough, dipping device and Cahn electrobalance

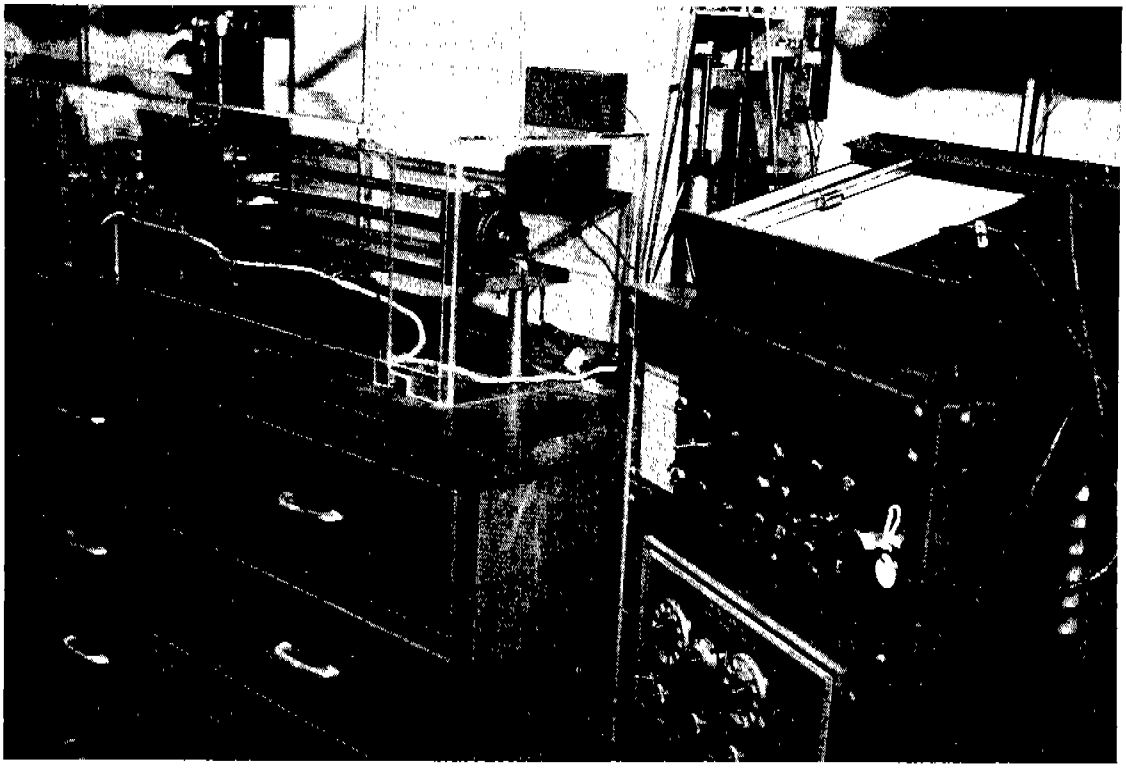

Figure 4 - View of hydraulic drive units, recorder and console

trough and has a platinum plate (1.5 cm x 1.0 cm x 0.0076 cm) suspended by silk thread from the B loop of the Cahn. The control unit and recorder are located in the control cabinet. The recorder is a Mosely model 7035A X-Y recorder which plots directly the surface pressure change of the film versus the distance the moveable-barrier has traveled.

The balance is used to measure pressure during force-area measurements and to actuate a pressure control device in constant pressure experiments. The maximum sensitivity in the pressure measurements is 0.04 dynes/cm.

The trough and electrobalance are housed in a solid Acrylite^{R} polymethylmethacrylate cage to protect it from dust or other air-borne particles and to maintain thermal equilibrium. This cage has an opening in the front which is covered with two flexible plastic sheets.

Constant temperature of the trough is maintained by a Lab-Line Instrument water circulating system and temperature control unit. This unit is located in the control cabinet. All runs in this study were made at a subphase temperature of 30° $\pm$ 0.1°C. The film balance is located in an air conditioned room where the temperature is maintained at 25° $\pm$2°C during all experiments. A constant water level can be maintained by establishing a siphon between a water source held at a constant level and the trough. Water is added continuously to the water source which as an overflow tube for removal of excess water above the desired level. It was generally found that it was not necessary to use this device since the water levels did not change appreciably during the times of the runs (usually 2 hours at a moveable-barrier rate of 19.2 cm/hour).

The dipping device is an automatic system for lowering and raising a solid support through the interface during the deposition of a monolayer. The device consists of three units: (1) the dipper and "slave unit" of the hydraulic drive, (2) the master hydraulic drive panel, and (3) the control box. The dipper and slave unit of the hydraulic drive are mounted in a supporting frame over the well in the trough. The dipper (rod and mounting clips for support material) is hydraulically driven up and down by the "slave unit." The master hydraulic drive panel consists of the oil cylinders and lines, the mechanical drive (the screw and synchronous motor) and two microswitches for cycling the dipping action. The control box has an off-on switch, momentary stop button, and indicator lights showing on, and the direction of movement, up or down.

The dipper moves at a rate of 0.2 cm/min. It automatically reverses direction and continues cycling until manually stopped.

The depth of dipping can be set by adjusting the microswitches on the master drive panel.

2. Procedure for Pressure-Area Measurements

1. Assemble apparatus and clean surfaces
   (a) Surfaces of the trough are cleaned with a detergent solution and repeatedly rinsed with warm water.

   (b) Barriers and machined edges of trough are cleaned with solvent (hexane) and rinsed with distilled-deionized water.

   (c) The platinum plate is first cleaned with benzene to remove any adhering polymer material. Then it is cleaned with chromate cleaning solution, rinsed carefully and flamed.

   (d) Distilled-deionized water or dilute salt solutions are added to the trough, and the surface of the water is swept (manually) five or six times with the Teflon-coated barrier by moving a barrier from one end of the trough to the other, pushing any contaminants on the surface over the end of the trough. On the last sweep the barriers are left at the end of the trough to trap any material not swept over the ends. The surface should be protected during this procedure from airborne contaminants.

2. Adjust water level and temperature.

3. Record Cahn readings.

4. Add 0.03 to 0.15 ml of a spreading solution containing the sample with a micrometer pipette. The solution should be added in a series of drops widely spaced over the surface. Concentration of sample in the spreading solution should be in the range 0.1-10.0 mg/ml. The amount of material added should cover 50% of the available area after compression into a monolayer.

5. Monitor any changes in pressure occurring during the addition of polymer solution, until there has been no change for 15 mins.

6. Using the constant-rate barrier drive, advance the barrier and record changes in pressure with changing areas.

3. Subphase Variation

The purpose of this phase of the study was to determine how various subphases affect the physical properties of the reconstituted lipoprotein films. This should indicate the conditions under which a film of optimum strength and toughness is obtained. A known amount of the solubilized red cell ghost material was added to the surface of aqueous solutions containing various inorganic salts. The material was allowed to reaggregate to form a film and this film was then compressed. These experiments were carried out on the automated film balance. A constant barrier sweep rate of 19.2 cm/hr. was used. The surface pressure - area plots for the compression of the films were displayed on an X-Y recorder. All films displayed the general features shown in Figure 5: a curve containing two generally linear portions, the slope of the second linear portion being greater than the first. The collapse pressure is a term used in this work to define that point where the extrapolated lines of the two linear portions of the curve intersect, as indicated in Figure 5. This is not a typical collapse pressure as observed with classical monolayers, e.g., stearic acid, but beyond this critical surface pressure these films display a marked difference in behavior on continued compression. For example, if the movable barrier is stopped in the area of the first linear portion of the curve, no change in film pressure occurs with time, but if the barrier is stopped in the area of the second linear portion of the curve, the pressure decreases with time, approaching but not falling below the above defined collapse pressure. These data have been interpreted to indicate that the film is still in an expanded state in the first part of the curve and hence is reversible and stable in this region. When the first linear portion of the π-A curve is extrapolated to zero pressure, the limiting area of the film is obtained, i.e., the area at which the total surface is covered with film. As the film is further compressed toward the collapse area, a smooth reorganization of the lipoprotein components appears to take place resulting in a uniform thickening of the film. The film thicknesses obtained at the collapse pressure (about 50-150 Å, see Table II) suggest that this structure is most nearly analogous to that of the intact cell membrane, whose thickness falls in the same range.[36] It is believed that beyond the collapse point, in the steeper portion of the π-A curve, the membrane is forced out of its planar state and either buckles out of the interface or forms overlapping layers. Finally, if the compression is carried out far enough a point is reached beyond which the pressure no longer increases with decreasing area, indicating that the film is now completely buckled. A similar behavior has been observed with monolayers of high M.W. polymers.[43]

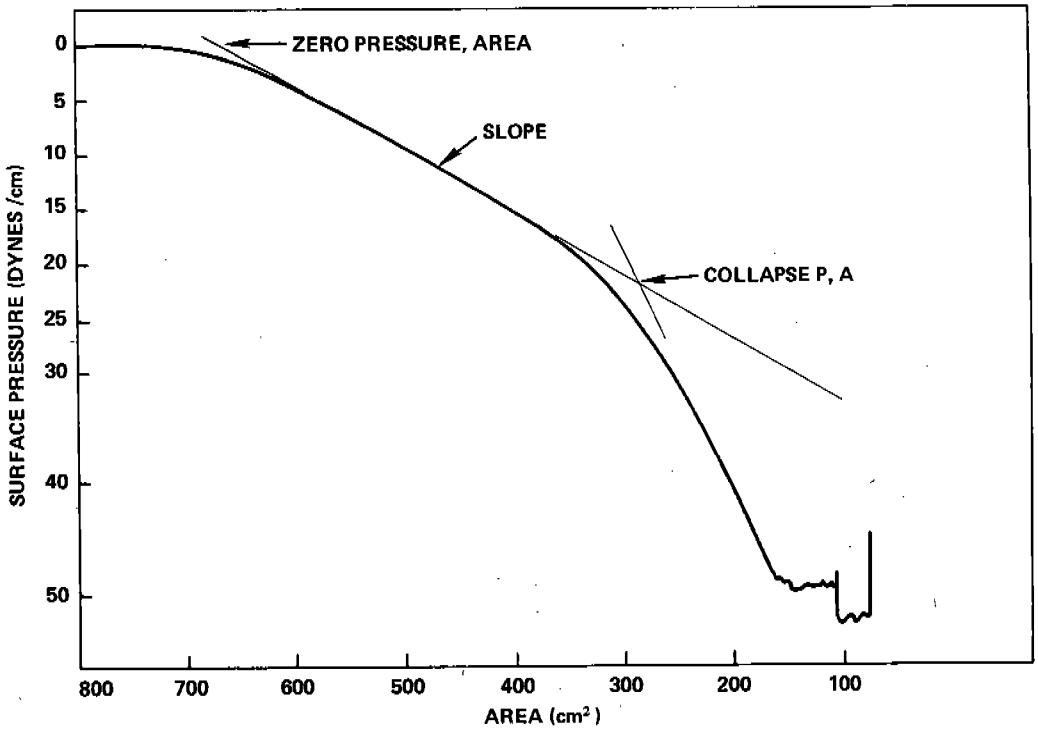

Figure 5 - π-A trace of lipoprotein film on 20 mM KCl, 5 mM Tris, pH 7.4, T = 30°C

The surface pressure-area curves of red cell lipoprotein films formed on a number of subphases have been obtained. Aqueous solutions of various mono-, di- and trivalent cations with several different anions have been used for the subphase at various pH's and ionic strengths. The data from these curves are presented in Tables II & III and the π-A curves are shown in Figures 6 and 7. In most cases reproducibility of the curves was checked by duplicate experiments. In cases of minor deviations, the results reported are averages of the duplicate runs.

i. Cations and Ionic Strength -

Critical examination of the data in Table II shows a remarkable consistency for the cation variation experiments at pH 7.4. Although six different cations were used over a 100-fold range of concentrations, the collapse pressures have fallen in the narrow range of 9-22 dynes/cm. Indeed, at constant ionic strength (μ = .02) subphases of the chlorides of Na^+, K^+, Li^+, Mg^{++} and Ca^{++} gave films with nearly constant collapse pressures (π_c = 18-22 dynes/cm.) The collapse areas and film thicknesses have likewise varied in a narrow range with no significant trends. The compressibility modulus, calculated from the slope of the reversible part of the curve, is typical of the values found for protein films in other work.[44] A most striking result has been obtained with La^{+++} at pH 4.8 (La^{+++} is not soluble at pH 7.4). A very high collapse pressure, a very small collapse area, and a correspondingly thick film is found with this ion. This may derive from the the trifunctional coordinating tendencies of the lanthanum ion with the phosphate groups of the phospholipids. The pressure-area curves of solubilized ghost preparations on the various cation chloride subphases are shown in Figure 6.

To the extent that it was investigated, ionic strength variation seems to have far less effect on the film properties than the specific nature of the cation in the subphase.

ii. Anions -

In marked contrast to the results with cation chlorides, when the subphase anion was varied, a striking effect on the collapse pressure was observed (Table III, Figure7). Use of the nitrate, sulfate and acetate salts of calcium at a concentration of 0.01M produced collapse pressures of 16.5, 23.5 and 33 dynes/cm., respectively, for the film compression. Since the collapse pressure over 0.01M $CaCl_2$

TABLE II

Effect of Subphase Cation and Ionic Strength Variation on π-A Curves

Subphase[a]	Collapse Pressure π_c (dynes/cm)	Collapse Area, $\pi = \pi_c$ (Å²/residue)[b]	Limiting Area, $\pi = 0$ (Å²/residue)	Thickness at $\pi = \pi_c$ (Å)[c]	Thickness at $\pi = 0$ (Å)[c]	Slope[d] $-d\pi/dA$ (dynes/cm³)	Compressibility Modulus $C_s^{-1} = -A \left(\frac{d\pi}{dA}\right)_{\pi=\pi_c}$ (dynes/cm)
1.0 M NaCl	9.5	9.60	18.7	49.8	25.5	0.020	8.2
10^{-1} M NaCl	20.5	5.76	17.3	83.0	27.6	0.063	15.4
2×10^{-2} M NaCl	22.0	3.86	15.5	124.0	30.7	0.050	8.25
2×10^{-2} M LiCl	20.5	7.61	17.6	62.8	27.2	0.050	16.3
2×10^{-2} M KCl	21.5	6.74	15.5	70.8	30.8	0.055	10.4
10^{-2} M $MgCl_2$	20.0	3.06	10.0	156.0	47.5	0.065	8.5
10^{-2} M $NiCl_2$	9.0	7.95	17.9	60.0	26.7	0.025	8.5
5×10^{-1} M $CaCl_2$	21.0	3.28	9.1	146.0	52.3	0.085	17.9
10^{-1} M $CaCl_2$	15.0	9.90	18.7	48.0	25.5	0.040	8.5
10^{-2} M $CaCl_2$[e]	22.0	3.52	16.0	120.0	30.0	0.080	13.6
10^{-2} M $CaCl_2$[f]	22.5	4.15	7.1	115.0	67.8	0.085	14.1
6.7×10^{-3} M $LaCl_3$[f]	32.0	1.89	10.5	252.0	45.3	0.097	7.9

a. All solutions contain 5×10^{-3} M Tris, pH 7.4. A constant amount (0.235 mg) of "soluble" material was added to each run. T = 30°C.
b. Average residue M.W. = 360.
c. Based on lipoprotein density of 1.15.
d. Slope of linear portion of curve between limiting area and collapse area.
e. These values are averages from over 20 experiments.
f. pH adjusted to 4.8 with concentrated HCl.

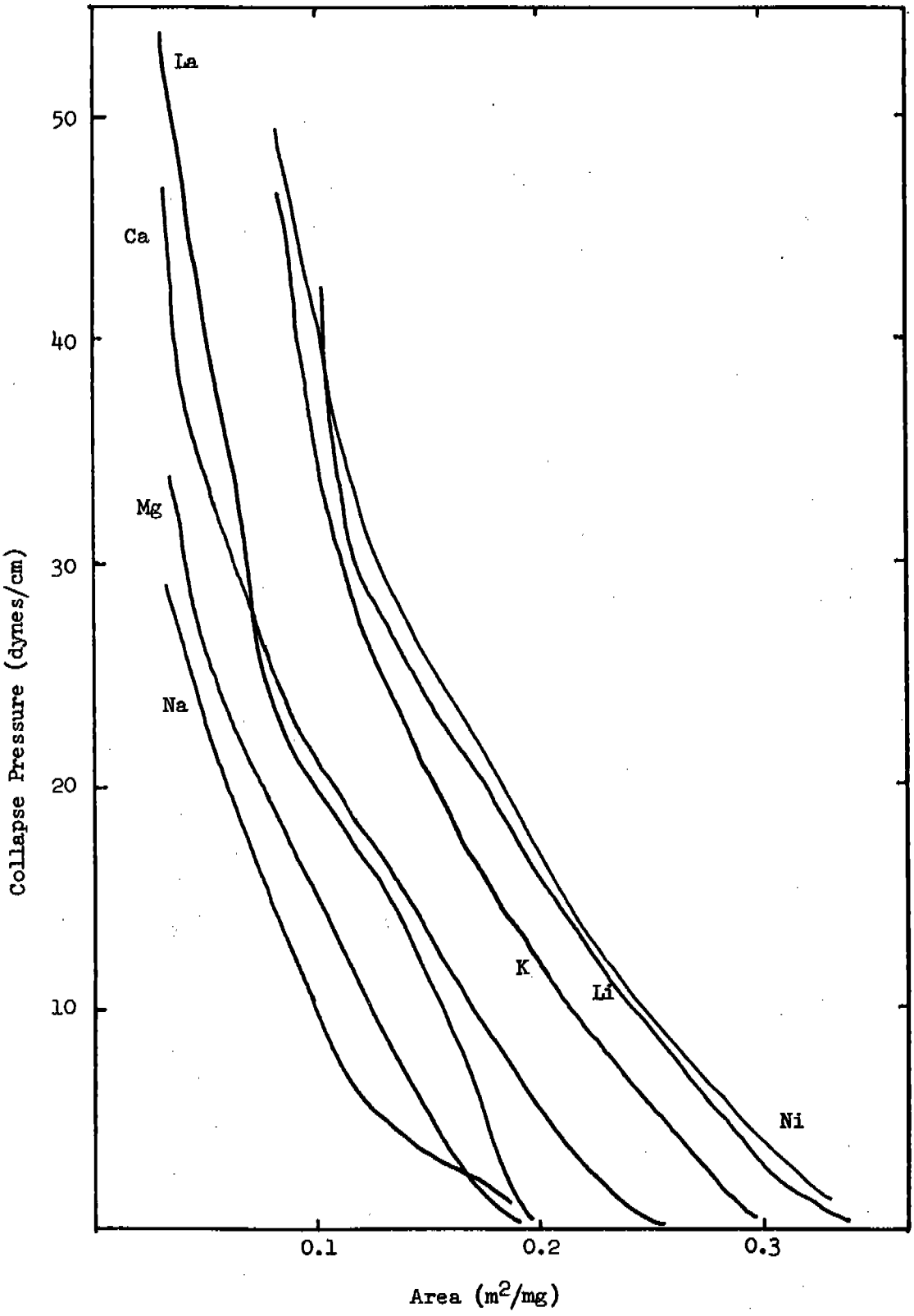

Figure 6 - Cation variation π-A plots

is 22 dynes/cm., it appears that the film strength is quite dependent upon the anion of the subphase, increasing with Ca salts in the order: $NO_3^- < Cl^- < SO_4^= < CH_3CO_2^-$. Complexing anions may tend to strengthen the membrane by crosslinking the metal ions, perhaps in much the same manner as the phosphate ester groups naturally present in the lipoprotein. The film areas at collapse and zero pressure are quite similar to those obtained earlier in the cation variation study. The other parameters, the calculated thickness of the film and the slope, are likewise unchanged.

It is apparent that all of these films are thicker than a monolayer since the area per residue at collapse is only 3-5 $Å^2$. The collapse areas per residue for phospholipid and protein monolayers are about 50-80 $Å^2$ and 20-40 $Å^2$, respectively.[45] It seems probable that the film is composed of globular lipoprotein complexes which are very compressible.

iii. pH-

In further film compression studies on our Langmuir trough, we have found that for 0.01 M $CaCl_2$ subphases the collapse pressure increases as the pH is increased or decreased from 7.4 as shown in Figure 8. However, this variation was not observable with KCl subphases on which the collapse pressure remained fairly constant when the pH was lowered as is seen in Table III.

iv. Glutaraldehyde Treatment -

When glutaraldehyde (0.05%) was added to the subphase after the film was formed at pH 7.4, it was found that the collapse pressure was considerably increased (see Table III). The stronger film is probably due to crosslinking of the amino groups of the proteins by the glutaraldehyde. It is interesting to note that the other film parameters such as the thickness, are essentially unaffected by the glutaraldehyde treatment. Hence the intact lipoprotein complexes are probably linked together by the glutaraldehyde via proteins on their surfaces. This treatment produced the strongest films of the soluble preparation, as judged by the collapse pressure, in the whole study.

It is difficult to explain the variation of film collapse pressure with salt subphase on a molecular basis. It seems likely that this variation is due to changes in the hydrophilic lipid-protein interactions within the complex.[46,47] These interactions are not well understood and are the subject of intense research in numerous laboratories.[48,49] Thus, the next step taken in this study was to isolate the membrane components, i.e., the lipids and proteins, and study the surface chemistry of each one separately and in various combinations.

TABLE III

Effect of Subphase Anion and pH Variation and Glutaraldehyde on π-A Curves

Subphase[a]	Collapse Pressure, π_c (dynes/cm)	pH	Collapse Area, $\pi = \pi_c$ [b] ($Å^2$/residue)	Area at Zero Pressure, $\pi = 0$ ($Å^2$/residue)[b]	Thickness at $\pi = \pi_c$ (Å)[c]	Thickness at $\pi = 0$ (Å)[c]	Slope[d] $-d\pi/dA$ (dynes/cm^3)
10^{-2}M $CaCl_2$	22.0	7.4	3.52	16	120	30.0	0.08
10^{-2}M $Ca(NO_3)_2$	16.5	7.4	4.1	13.1	116	36.5	0.06
10^{-2}M $Ca(OAc)_2$	33.0	7.4	4.8	16.7	100	28.5	0.06
10^{-2}M $CaSO_4$	23.5	7.4	4.6	18.7	104	25.5	0.05
10^{-2}M $CaSO_4$	28.3	2.0	4.7	18.7	102	25.5	0.045
10^{-2}M $CaCl_2$[e]	35.5	7.4	3.84	16.0	125	29.8	0.05
2 x 10^{-2}M KCl	24.9	7.4	6.74	15.5	70.8	30.8	0.055
2 x 10^{-2}M KCl	23.6	4.8	5.10	11.5	94	41.5	0.105
2 x 10^{-2}M KCl	19.8	2.0	6.28	18.7	76	25.5	0.03
10^{-2}M $CaCl_2$	25.3	9.0	3.75	12.7	127	37.8	0.095
10^{-2}M $CaCl_2$	24.7	4.8	4.15	7.1	115	67.8	0.08
10^{-2}M $CaCl_2$	27.9	2.0	4.1	18.7	116	25.5	0.06

a. All contain 5 x 10^{-3}M tris. A constant amount (0.235 mg) of "soluble" material was added in each run. T = 30°C.
b. Average residue MW = 360.
c. Based on lipoprotein density of 1.15.
d. Slope of linear portion of curve between limiting area and collapse area.
e. 0.05% glutaraldehyde added to subphase after film was formed.

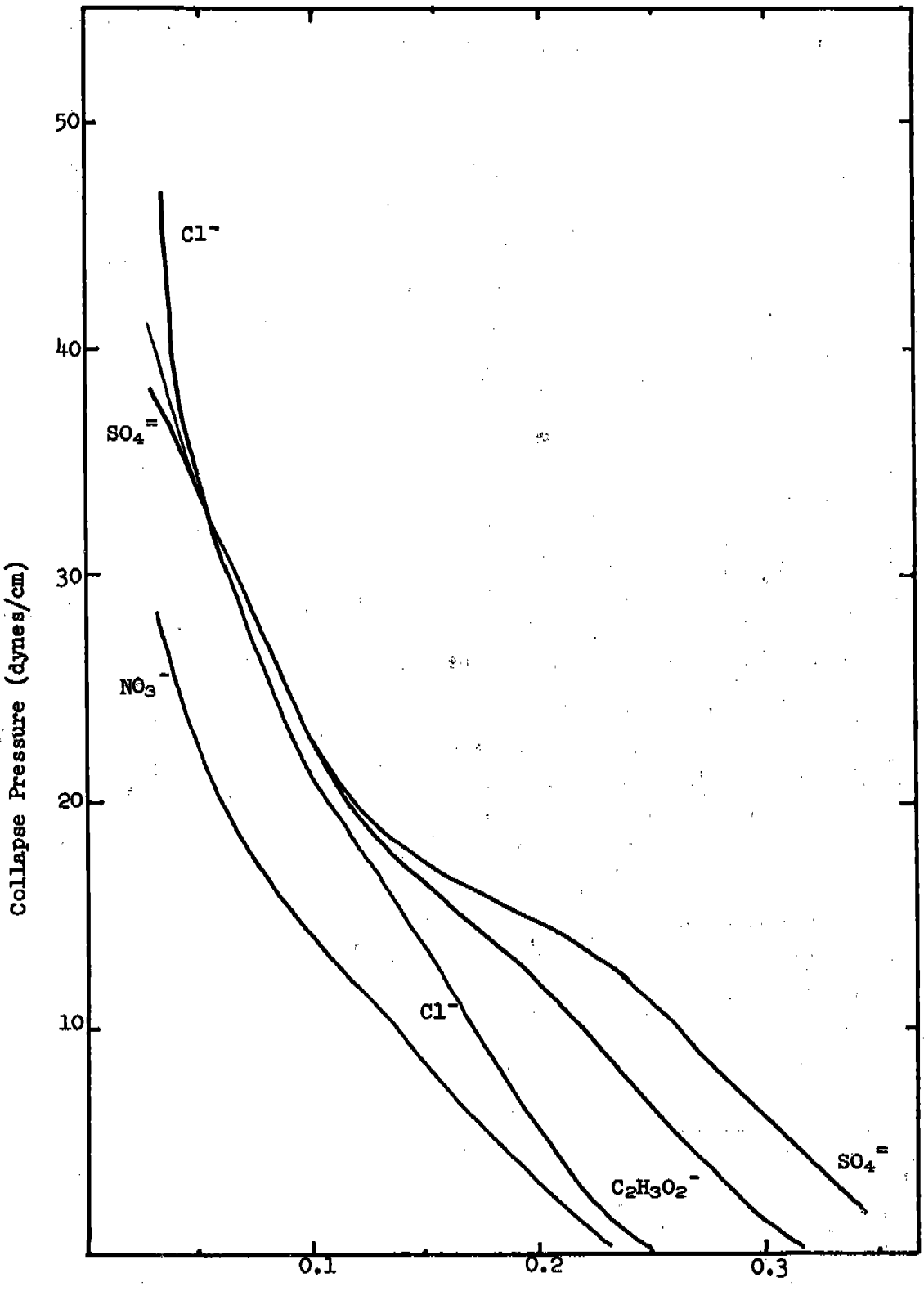

Figure 7 - Anion variation π-A plots

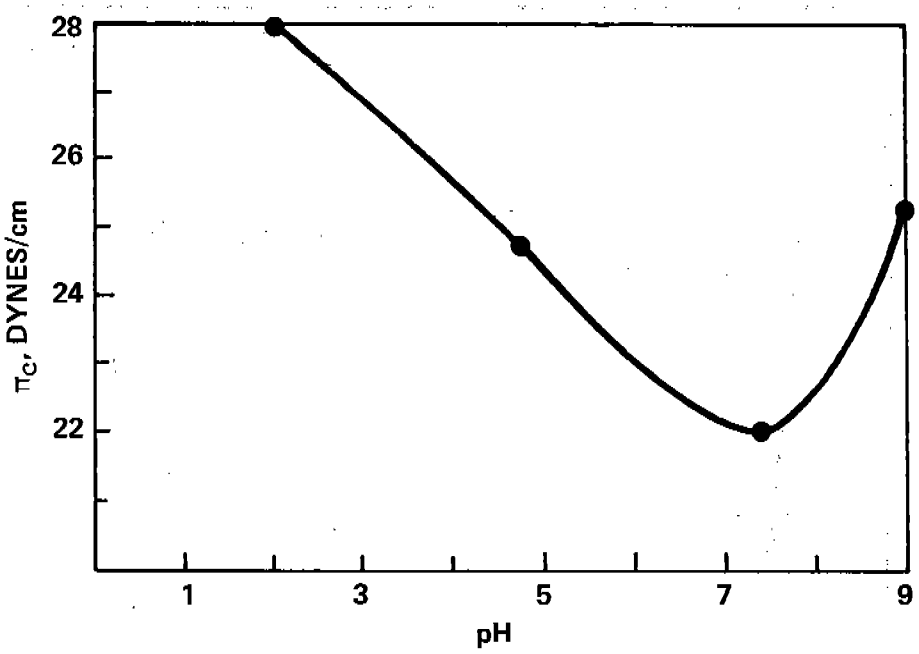

Figure 8 - Variation of lipoprotein film collapse pressure with pH over Ca

4. Isolated Lipids and Proteins

Techniques for the separation of lipid and protein components were developed so that films from each of these species could be studied on the Langmuir trough. It was hoped that such studies would determine which of these components is contributing most to the strength and integrity of the lipoprotein film.

For these experiments, two methods of isolating the protein components from the erythrocyte membrane were used, one of which utilized pyridine and the other butanol for solubilization. More than one method was used for the protein isolation because of the known polydispersity of the membrane proteins[50-52] and the numerous claims that different protein fractions are obtained from different isolation procedures.[38,40,53-56] The degree of lipid contamination in the protein was another factor for the surface studies. The "pyridine" protein contained about 6% phospholipid, the "butanol" protein less than 1%. The membrane lipid was isolated by the standard chloroform-isopropanol procedure. These methods are outlined in detail in Sections IIC and IID.

The approach we have taken is to determine the surface pressure-area curves under standard conditions (10^{-2} M $CaCl_2$ subphase, pH 7.4) of (1) each of the protein and lipid components in a similar ratio to that in the membrane lipoprotein and with intact ghost films. To further elucidate the surface chemistry of these systems, π-A curves were also run on several pure model proteins (human serum albumin, casein, cytochrome-C, and insulin) and mixtures of these with the extracted membrane lipid. The results of these experiments are collected in Table IV and the π-A curves are traced out in various combinations in Figures 9-13.

Films prepared from the pyridine solubilized protein show properties similar to those of the reconstituted lipoprotein preparation and intact ghosts; however, the isolated protein forms a thinner, somewhat more expanded film (Figure 9). The butanol solubilized protein, on the other hand, displays a far steeper slope for the π-A curve, indicative of a less expanded film (Figure 10). It is reasonable to assume that, since much protein remains insoluble in both procedures, the butanol solubilization removes a slightly different fraction or induces more protein aggregation than the pyridine procedure, thus giving rise to different curves. It is interesting to note, however, that their collapse pressures are quite close despite differences in curve shape. It would appear that the two-dimensional film strengths of the two protein extracts are very similar in spite of differences in film thickness. This may arise from tighter coiling and a higher degree of aggregation in the butanol preparation.

Table IV.

Data from π-A Plots of Isolated Proteins and Lipids and their Mixtures[a]

System	Collapse Pressure, π_c (dynes/cm)	Slope $-d\pi/dA$ (dynes/cm^3)	Collapse Area, $\pi=\pi_c$		Limiting Area, $\pi=0$		Thickness (Å)[b]	
			m^2/mg	Å^2/residue	m^2/mg	Å^2/residue	$\pi=\pi_c$	$\pi=0$
Red cell ghosts	26.5	0.100	0.065	3.56	0.182	10.0	135	47.6
Soluble lipoprotein preparation	22.0	0.080	0.072	3.52	0.149	16.0	120	30.0
Extracted lipid[c]	25.0	0.170	0.250	20.7	0.350	28.4	48.5	27.0
Phosphatidyl ethanolamine	33.0	0.220	0.510	42.6	0.690	67.5	19.0	14.0
Pyridine extracted membrane protein	31.0	0.120	0.160	2.70	0.385	6.5	49.2	20.4
Recombined pyridine protein + lipid	30.0	0.105	0.085	4.68	0.210	11.7	102	20.4
Butanol extracted membrane protein	29.0	0.230	0.064	1.03	0.092	1.5	124	85.5
Recombined butanol protein + lipid[d]	43.0	0.220	0.018	4.08	0.050	11.4	494	176
Human Serum Albumin	11.0	0.100	0.290	4.80	0.550	9.1	34.8	18.2
Albumin + lipid	11.0	0.085	0.190	4.00	0.510	8.0	51.5	38.6
Whole Casein[e]	28.0	0.100	0.105	1.80	0.390	6.6	90.0	27.0
Casein + lipid	21.5	0.060	0.089	4.37	0.242	11.8	107	87.0
Bovine pancreas insulin[f]	9.0	0.145	0.215	3.60	0.270	4.6	46.4	37.2
Insulin + lipid	3.5	0.060	0.157	7.70	0.200	9.8	63.5	50.0
Horse heart cytochrome-C[g]	11.0	0.100	0.115	1.95	0.225	3.8	87.0	44.2
Cytochrome-C + lipid	10.0	0.130	0.045	2.18	0.079	3.9	213	121

a. Each film was formed with 0.08 - 0.5 mg of indicated material on a subphase of 10 mM $CaCl_2$, 5 mM Tris, pH 7.4. T = 30°C. All recombined additives or mixtures were in a 1:1 (wt/wt) ratio.

b. Based on the following densities:
lipoprotein d = 1.15 g/cc;
membrane protein d = 1.27 g/cc;
membrane lipid d = 1.03 g/cc;
other proteins d = 1.00 g/cc;
other proteins + lipid d = 1.015 g/cc

c. Contained 12.5% 2-Propanol.
d. Recombined in a 3.8:1 (wt/wt) ratio.
e. Contains 10 mM KCl, 2 mM NaOH, pH 11.0.
f. Contains 0.1 M KCl, pH 7.5.
g. Contains 0.1 M $KC_2H_3O_2$, pH 6.8.

Adding extracted lipid back to the pyridine protein results in a curve which is nearly identical to that of the reconstituted lipoprotein (Figure 9). The general shape of the curve obtained from combination of butaonol protein and lipid was little changed from the protein curve, except for an increase in the collapse pressure (Figure 10). The reconstituted pyridine protein and lipid system seems to be the better model for the lipoprotein.

The surface pressure-area curves of the extracted lipids were similar to those of commercial lipids, e.g., phosphatidyl ethanolamine (see Table IV), but were strikingly different from the membrane protein and lipoprotein curves. The lipid films generally achieved a stable surface pressure of 25 dynes/cm, beyond which further compression failed to increase the film pressure.

In further attempts to characterize the membrane proteins, several model proteins were examined. These included human serum albumin, whole casein, bovine pancreas insulin, and horse heart cytochrome-C. Of these, casein gave a π-A curve which was quite similar to that of the pyridine extracted protein (Figure 11). The general shape of the curve obtained from cytochrome-C was also similar to the membrane protein with the exception that the former gave a much weaker film. To complete the study, each of the model proteins was combined with the extracted membrane lipid and compressed at the air-water interface. Consistently, the added lipid decreased the collapse pressure, lowered the collapse area, and increased the film thickness, but did not otherwise change the shape of the curve (Table IV and Figures 12 and 13).

B. Circular Dichroism

A new analytical technique that has recently been applied to conformational studies of proteins is circular dichroism (CD).[57] The secondary structure of proteins and polypeptides may exist in three principal conformations: (1) α-helix, (2) β pleated sheet, and (3) random coil. The contribution of each of these conformations to the total protein structure can be determined by measuring the CD spectrum and comparing it with CD spectra of proteins having known amounts of these three structures. For example, polylysine with 100% α-helix, 100% β or 100% random coil structures has been prepared and its CD spectra analyzed.[58] CD spectra of proteins are characterized by four principal bands: (1) an $n-\pi^*$ helical band with a negative minimum at about 223 mμ, (2) an unordered transition with a negative maximum around 214 mμ, (3) a $\pi-\pi^*$ transition polarized paralled to the helix axis with a negative minimum at about 208 mμ, and (4) a $\pi-\pi^*$ transition polarized perpendicular to the helix axis with a positive maximum at about 195 mμ.[59]

Several recent CD studies of biological membranes have been

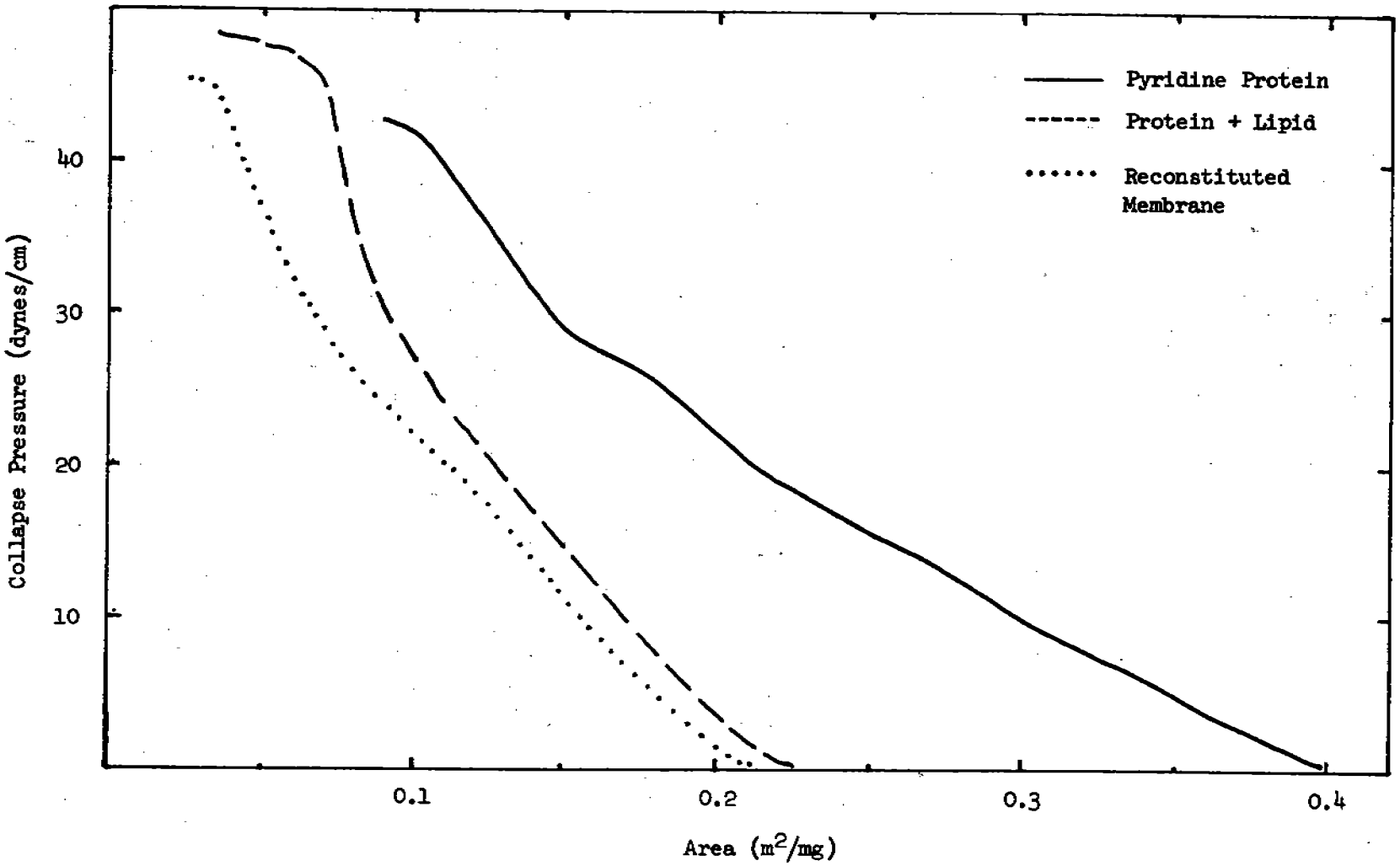

Figure 9 - π-A plot of pyridine extracted protein systems

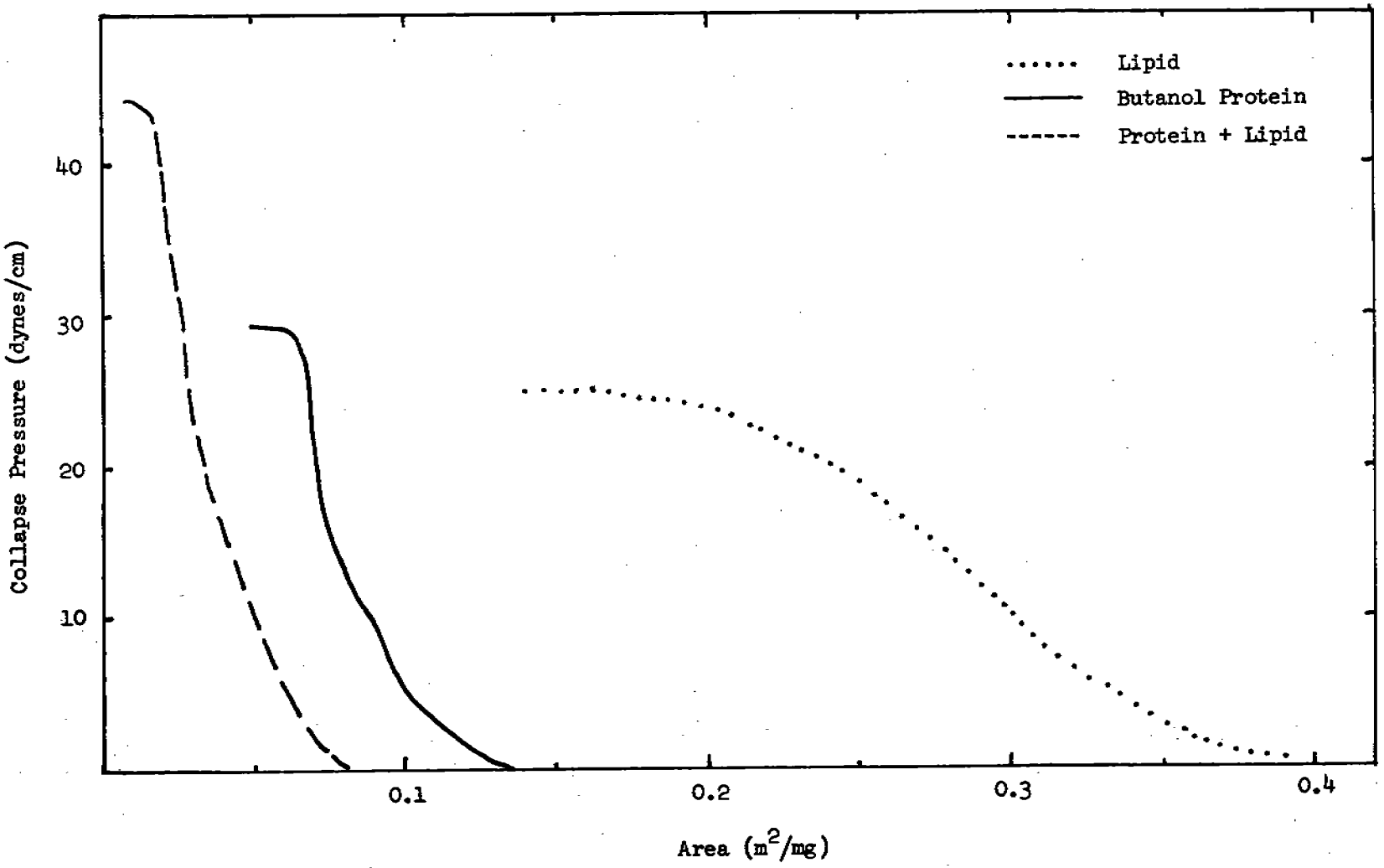

Figure 10 - π-A plot of butanol extracted protein systems

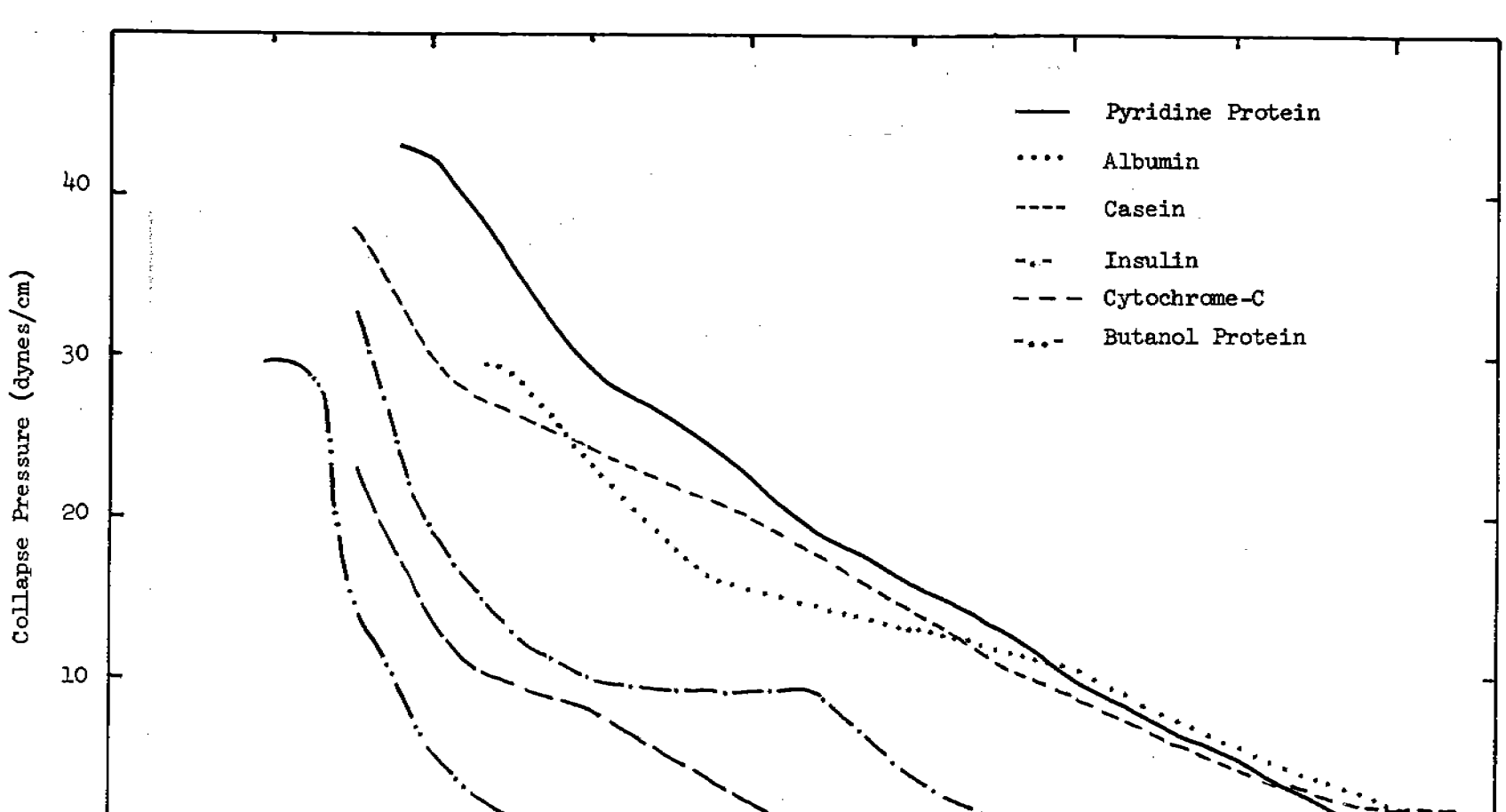

Figure 11 - π-A plots of membrane and model proteins

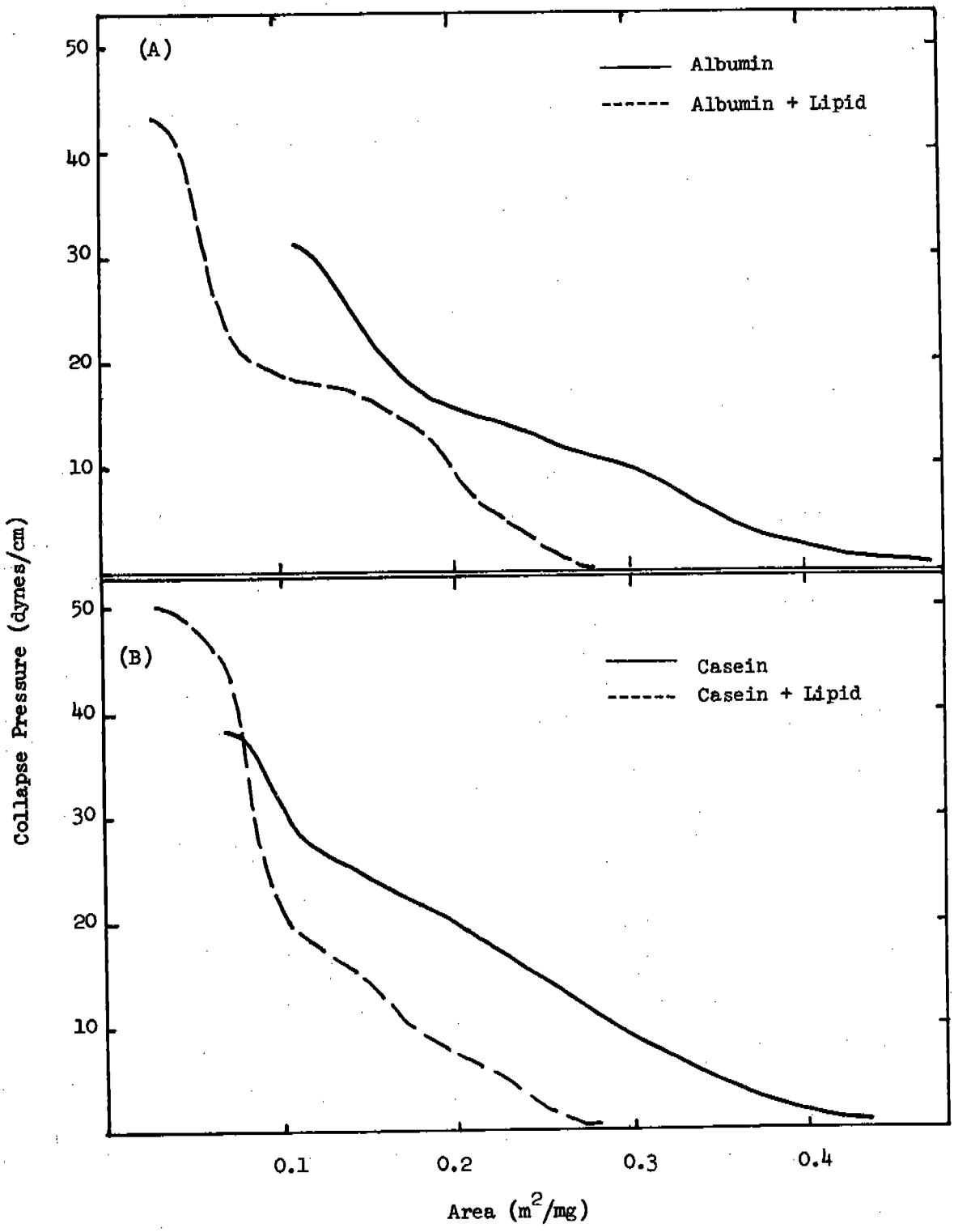

Figure 12 - π-A plot of (A) human serum albumin and albumin-lipid solutions; (B) whole casein and casein-lipid solutions

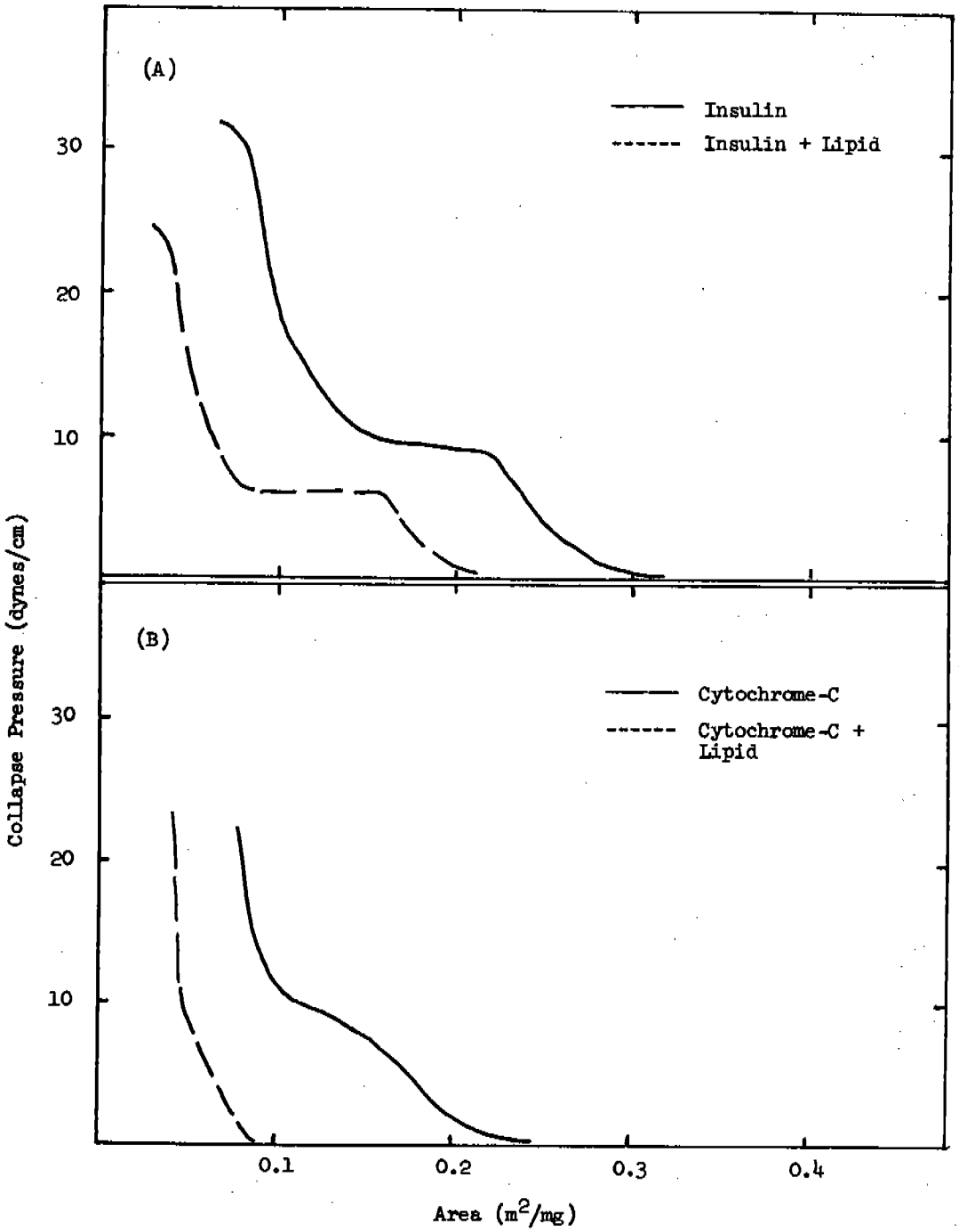

Figure 13 - π-A plot of (A) bovine pancreas insulin and insulin-lipid solutions; (B) horse heart cytochrome-C and cytochrome-C-lipid solutions

reported [59-66] and it is generally concluded that the bulk of the membrane protein is in the α-helix conformation. It was our objective in this CD study of erythrocyte membranes to determine what changes, if any, occur in the secondary structure of the membrane protein when ghosts are solubilized and reconstituted and when the protein is isolated from the membrane and again recombined with membrane lipids. Lipid molecules do not contribute to the CD spectrum. The CD spectra obtained are shown in Figures 14 and 15 and data from these curves is given in Table V.

Figure 14a shows the curves of ghosts, soluble preparation and reconstituted membranes. The great similarity of the curve shapes indicates that no significant changes in protein secondary structure have occurred upon sonication of the ghosts and the subsequent reconstitution with Ca. The slight blue shift of the 209 and 192 mμ bands after solubilization may be attributed to an increase in the polar environment of the free lipoproteins. The intensity changes after solubilization are probably due to scattering effects. The return of the reconstituted membrane spectrum to a virtual identity with that of the original ghosts is highly significant and adds further support to our earlier conclusions[34] that these two membranes possess the same structure.

The spectra of the pyridine and butanol isolated proteins shown in Figure 14b are again very similar to those of the intact ghosts and demonstrate that no changes in protein secondary structure, such as denaturation, have accompanied their release and isolation from the membrane. However, when extracted lipid was added back to these isolated protein fractions the resulting curves did not revert to the intact ghost spectra. Instead, blue shifts in the 210 and 190 mμ bands again occurred indicative of an increasing polar environment. Perhaps the added phospholipids interacted with the isolated protein via the polar phosphate groups instead of the nonpolar association of the hydrocarbon tails of the lipids with the nonpolar side chains of the proteins. The latter is believed to be the major lipid-protein interaction mechanism in biological membranes.[59,67-70]

Based on these results, a highly important conclusion can be reached regarding the association of lipids and proteins in the red cell membrane. There appears to be an unique interaction between the small lipid molecules and the macromolecular membrane proteins present in the intact membrane. When these lipoprotein complexes are broken up by treatment with organic solvents or detergents, this special lipoprotein structure cannot be readily regenerated by combining the isolated lipid and protein components. However, as long as the lipoprotein complexes remain intact, the original membrane structure can be reversibly disaggregated into its individual lipoprotein subunits by physical treatment such as sonication, and subsequently reconstituted by treatment with metal ions to regain its former structure. Presumably these lipoproteins have a very special tertiary structure which is irretrievably lost when the lipid is removed. The only other possibility is that in our protein extraction procedures we failed to isolate a special template protein around which the membrane lipoprotein structure is constructed.

Table V.

Circular Dichroism Data for Membrane and Model Proteins

System	Band Positions (nm)					
	$\perp\pi\rightarrow\pi^*$	$\parallel\pi\rightarrow\pi^*$	unordered	$n\rightarrow\pi^*$	% α-helix[a]	π_c[b] (dynes/cm)
Ghosts	191	209	215	221	15.5	22
Sonicated ghosts	190	207	211	220	33.0	22[c]
Reconstituted membranes	192	210	215	221	13.4	22
Butanol extracted protein	190	208	212	216	41.2	29
Recombined butanol protein + extracted lipid[d]	-	210	217	220	- [e]	43
Pyridine extracted protein	190	211	215	221	36.2	31
Recombined pyridine protein + extracted lipid	191	211	217	220	-	30
Human serum albumin	190	208	215	220	52.0	11
Albumin + lipid	-	208	212	218	-	11
Whole casein	-	200	220[f]	220[f]	12.0	28
Casein + lipid	-	200	220[f]	220[f]	-	22
Bovine pancreas insulin	-	210	218	223	20.6	9
Insulin + lipid	-	208	224	226	-	3.5
Horse heart cytochrome-C	197	210	214	220	11.0	11
Cytochrome-C + lipid	-	-	-	-	-	10

a. Calculated from the following equation[58]: $\alpha\text{-helix} = \dfrac{[\theta]_{208}-4000}{33000-4000}$

b. Data from Table III.

c. 2×10^{-2} M NaCl substituted for the $CaCl_2$ in subphase.

d. All recombined solutions and protein-lipid mixtures contained 12.5% iso-propanol. The proteins and lipid are in a 1:1 (wt/wt) ratio.

e. No α-helix content was calculated for 2-component systems.

f. Approx. point of inflection.

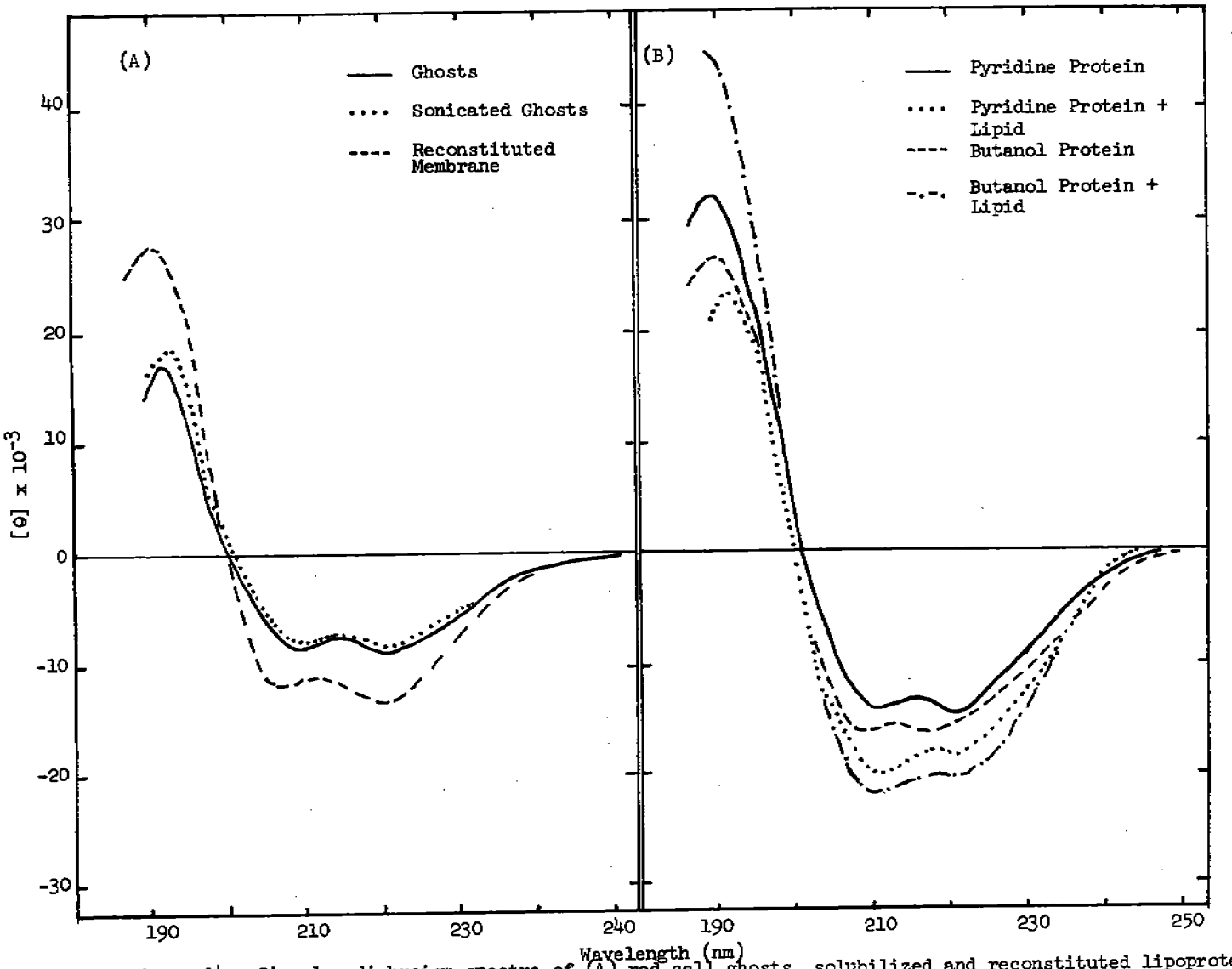

Figure 14 - Circular dichroism spectra of (A) red cell ghosts, solubilized and reconstituted lipoprotein preparations and (B) extracted proteins and protein-lipid systems

However, this seems rather unlikely since the same result was found for protein extracted by two different methods.

In order to compare the results for membrane protein with the behavior of a group of model proteins, CD spectra were obtained for albumin, casein, cytochrome-C and insulin before and after the addition of extracted lipid. These curves are presented in Figure 15 and the data is collected in Table V. The albumin and cytochrome-C spectra indicate the presence of a high α-helix content; the casein and insulin are mostly in the random coil form. Relatively little effect is noted on adding lipid to each of these proteins. There is at most a slight blue shift in the bands near 208 and 220 mμ, much the same result as was found with the membrane protein. It can thus be concluded that addition of lipid to aqueous solutions of proteins does not result in any significant change in the secondary protein structure and what changes that do occur in the tertiary structure result from polar interactions with the lipid.

One final observation can be made by comparing the CD spectra of these proteins with their film collapse pressures. It has been noted by Evans et al [71] that the tendency of a protein molecule to unfold at an interface and generate a high surface pressure is an inverse function of the degree of folding or tertiary structure in the protein. For example, casein, which has little tertiary structure, has a high surface activity, whereas lysozyme, with a strong tertiary structure, does not form a dilute highly spread film on water. Albumin, with a high helical content and a moderate degree of tertiary structure displays an intermediate tendency to form a dilute film. Therefore, it would appear that the secondary structure of a protein plays little role in its surface activity.

We have shown that the surface activity of red cell ghosts is comparable to that of the solubilized membrane lipoprotein. Therefore, little if any change in the tertiary structure of the globular lipoproteins has taken place upon sonication. In the isolated protein studies, the lack of dependence of surface activity on the secondary structure is revealed by comparing cytochrome-C, casein and the membrane protein. Cytochrome-C and membrane protein have very similar α-helix content from their CD spectra, but do not form similar films; on the other hand, films of casein and pyridine protein are almost identical whereas casein is 100% random coil and the pyridine has a very high helical content.

Addition of lipid had very little effect on the collapse pressures of the membrane protein or most model proteins, suggesting minimal change in the tertiary protein structure had been induced by lipid. Lipid invasion of the spread protein monolayer was evident, however, from the increase in film thickness, but these lipid-protein interactions must be fairly weak. It may be merely chance that the π-A curve for the lipid plus pyridine protein nearly coincided with that of the solubilized lipoprotein, since the protein tertiary structure appears to be very different in these two systems as judged by CD. There is obviously a higher preference for the extracted lipid

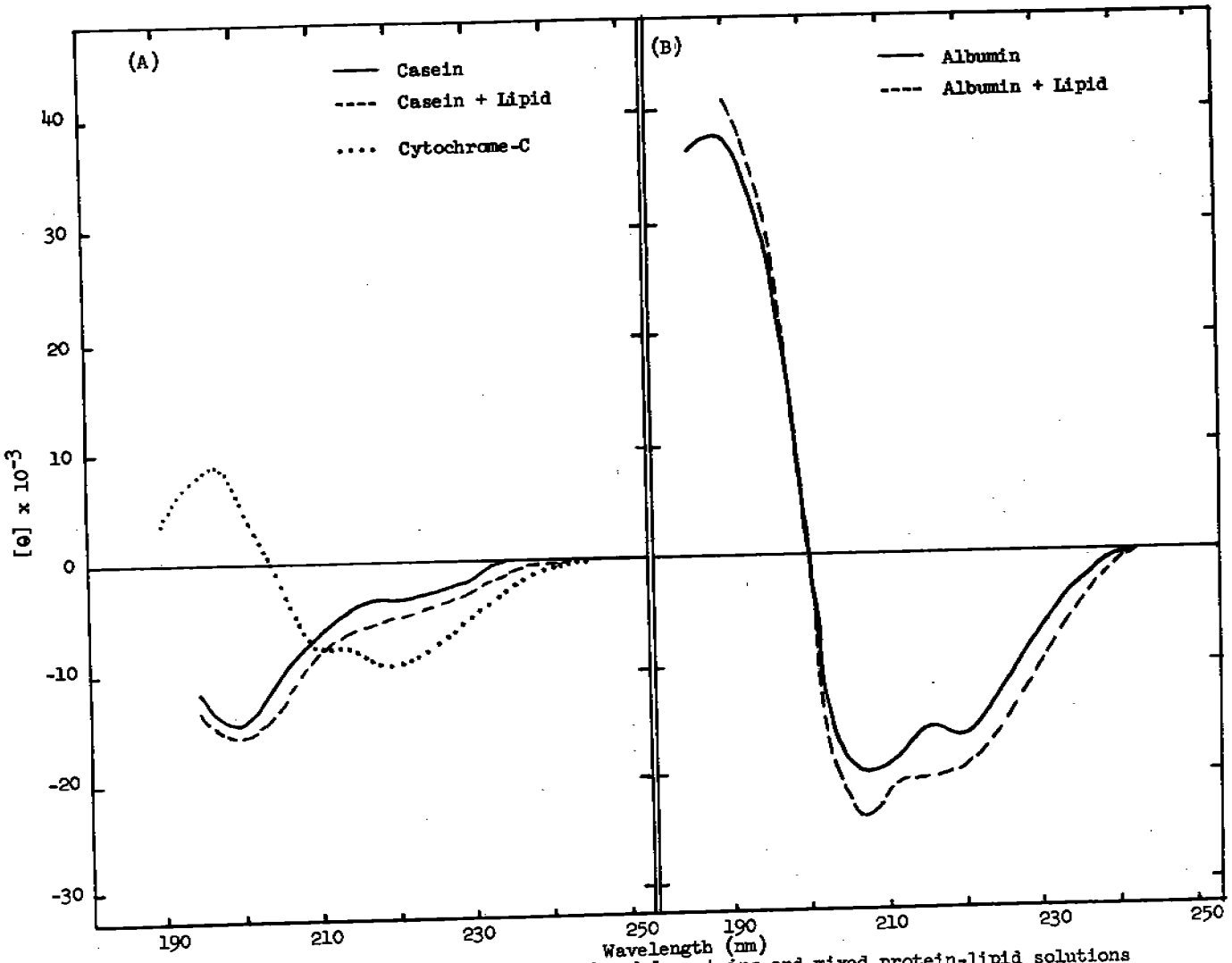

Figure 15 - Circular dichroism spectra of model proteins and mixed protein-lipid solutions (A) casein and cytochrome-C; (B) albumin

displayed by the membrane protein than by the other proteins in the study, but a complete understanding of the effect of tertiary structure of membrane protein on the biochemical and biophysical functional characteristics, and especially the permeability, of the red cell membrane must await further study.

C. Gel Electrophoresis

In order to get at the question of possible protein fractionation during the isolation procedures using pyridine and butanol a very brief attempt was made to resolve the protein components by gel electrophoresis.[72] This technique has been used extensively to resolve membrane proteins.[50] A Buchler Disc Electrophoresis apparatus was used. The protein sample was placed on 5% polyacrylamide gel in 0.1 M phosphate buffer (pH 7) containing 0.1% sodium dodecyl sulfate and electrophoresis was carried out at 50V (75 ma) for two hours, using cytochrome-C as a visual marker. The proteins were fixed with sulfosalicylic acid, stained with Amido Black and destained in 7% acetic acid at 100V. Molecular weight markers used were insulin (6,000), cytochrome-C (12,000), whole casein or chymotrypsin A (25,000), ovalbumin (45,000), and aldolase (158,000).

The samples studied were (1) red cell ghosts, (2) soluble sonicated preparation, (3) butanol extracted protein, and (4) pyridine extracted protein. Only qualitative results were obtained. The ghosts and sonicate gave quite similar patterns with the strongest bands being two sharp ones, close together, in the 150-250,000 MW range. There was relatively little low MW, so-called mini-protein[73] in these samples. The pattern for the pyridine protein was similar to that of the ghosts and sonicate, including the two strong high MW bands, but a large portion, perhaps as much as 50%, of the protein did not migrate at all, indicative of high degree of aggregation. The butanol protein was quite dilute and gave only a diffuse band pattern which could not be compared with the others.

The only tentative conclusion that can be drawn from these electrophoresis experiments is that there does not appear to be any significant fractionation of either high or low MW protein components during either pyridine or butanol isolation. It is still possible that unique structural proteins were not extracted. These could not be identified without a more extensive quantitative electrophoretic study. The failure to find large amounts of mini-protein of MW about 5000 suggests that the protein isolation methods used by us were mild and did not produce severe disaggregation.

IV. PERMEABILITY STUDIES

The primary objective of this project is to develop data on the permeability characteristics of the reconstituted lipoprotein films prepared from solubilized red cell membranes. The surface studies in the preceding section have clearly established the fact that continuous films of very large area can be prepared on still water surfaces. These films are very strong by comparision with the usual monolayer films; however, because of their

extreme thinness (ranging from 50 to 300 Å in thickness) it is impractical to manipulate or use these films unless they are supported. Of course, their thinness could be a decided advantage in their use as permselective membranes, provided a suitable porous support was found for coating. If this effort was not successful, we planned to incorporate the reconstituted lipoprotein preparation into the pores of preformed membranes or even cast polymer membranes from formulations containing the lipoprotein.

Several preliminary attempts were made to layer the unsupported films between two aqueous phases in order to make flux or conductance measurements directly on the film, analogously to the recent studies on phospholipid bilayer membranes. However, in spite of the use of water phases of differentdensity and special double chambered cells, no evidence for a salt barrier across the membrane was found and there is some doubt that a continuous membrane between the two water phases was maintained. The greater hydrophilicity of the lipoprotein as compared with the lipophilic phospholipid bilayer systems[1] probably accounts for this.

A. Coating Porous Supports

Our initial approach to coating the lipoprotein membrane on porous supports was to use the Langmuir-Blodgett dip coating technique.[74] The plan was to coat several trial surfaces with one or more layers of lipoprotein film from an air-water interface by dipping the support through the film and into the subphase.[45] Our film balance is equipped with an automatic dipping device for this purpose which was described in Section III A-1. The first surface to be tested for its ability to pick up the film was glass. Glass microscope slides were cleaned with dichromate cleaning solution and phosphoric acid to give a very hydrophilic surface. In a second experiment the glass slides were coated with a silicone concentrate to produce a hydrophobic surface. However, both types of surfaces appeared to give the same result during the dipping process: the supports were always coated only on the upstroke of the dip. After each dip cycle the films adhering to the support were either air dried or dried in a gentle stream of nitrogen. This drying procedure was necessary for more than one layer of film to be coated on the support. If the coated support was not dried after each dip the film would come off on the downstroke of the subsequent dip.

If the supported films were dried in an oven for 5-10 minutes at 100°C the support would then pick up film both on the up and down stroke of the dip. This behavior was different from that observed in the air or nitrogen drying technique where the support would only pick up film on the upstroke.

It seems that the drier the lipoprotein film is the more strongly it adheres to itself. This contrasts with the behavior of simple monolayers of stearic acid or phospholipids with which polar sides of the film adhere to polar surfaces only and the nonpolar sides to nonpolar surfaces only.[45] The experiments with the polar and nonpolar glass surfaces also indicate that the surfaces of the lipoprotein films are not strongly polar

or nonpolar, but possibly amphoteric.

The uptake of the lipoprotein films during the coating of porous supports was demonstrated both qualitatively in a small Teflon-lined cake pan where talc was sprinkled on the film and quantitatively in the Langmuir trough where the dipping was carried out at a constant film pressure of 10 dynes/cm. Using a millipore filter (Pellicon, PSAC) as a porous support, eight layers of the film were applied at constant pressure. The area taken up per dip, which was recorded on the X-Y recorder, was consistantly 10-15% higher than the area of the filter. This may be due to some coating on the downstroke or nonuniformity during the coating on the upstroke. The films were dried after each cycle in a stream of nitrogen.

The presence of the lipoprotein films on the supports could also be demonstrated directly by attenuated total reflectance (ATR) infrared spectroscopy. Initial spectra were obtained for films on glass or metal plates. The best spectrum was obtained when the film was directly coated onto both sides of the Irtran ATR plate itself. The major bands observed were at 3370, 2940, 1740, 1650, 1600, 1500, 1300, 1070 and 640 cm^{-1}. The characteristic infrared absorptions of phospholipids occur at 1070 cm^{-1} (POC bonds) and at 1740 cm^{-1} (ester group). The major absorption assignable to the protein component is that due to its amide groups and this band comes at 1650 cm^{-1}.[75] Thus, by this method we have a means of determining, at least qualitatively, the composition of the films that have been coated onto a support. This method works better for films coated on glass than on cellulosic supports since the cellulose absorptions tend to obscure the protein and phospholipid bands.

B. Direct Osmosis Experiments

After the first Millipore filters (Pellicon, type PSAC) were successfully coated with the reconstituted red cell lipoprotein film, many attempts were made to measure the resistance of the films. In these experiments, the coated filters were mounted either alone or together with a second uncoated filter between two osmotic cell chambers both filled with the same NaCl solution (range 10^{-3}-1M) and the resistance was measured across the membrane with two platinized platinum electrodes. There was only a very small difference between the resistance of the coated and uncoated filters which would indicate that the film is quite permeable to NaCl, or that it is not continuous.

These films are extremely thin (100 Å or less) and fragile, but it was hoped initially that they could be coated on to supports as a continuous film. However, this appears not to be the case. Early experiments in which Millipore filters (Pellicon PSAC) were dip-coated with up to 50 total coats of the lipoprotein film (coating both sides of the filter) showed little change in the salt flux of coated versus uncoated supports.

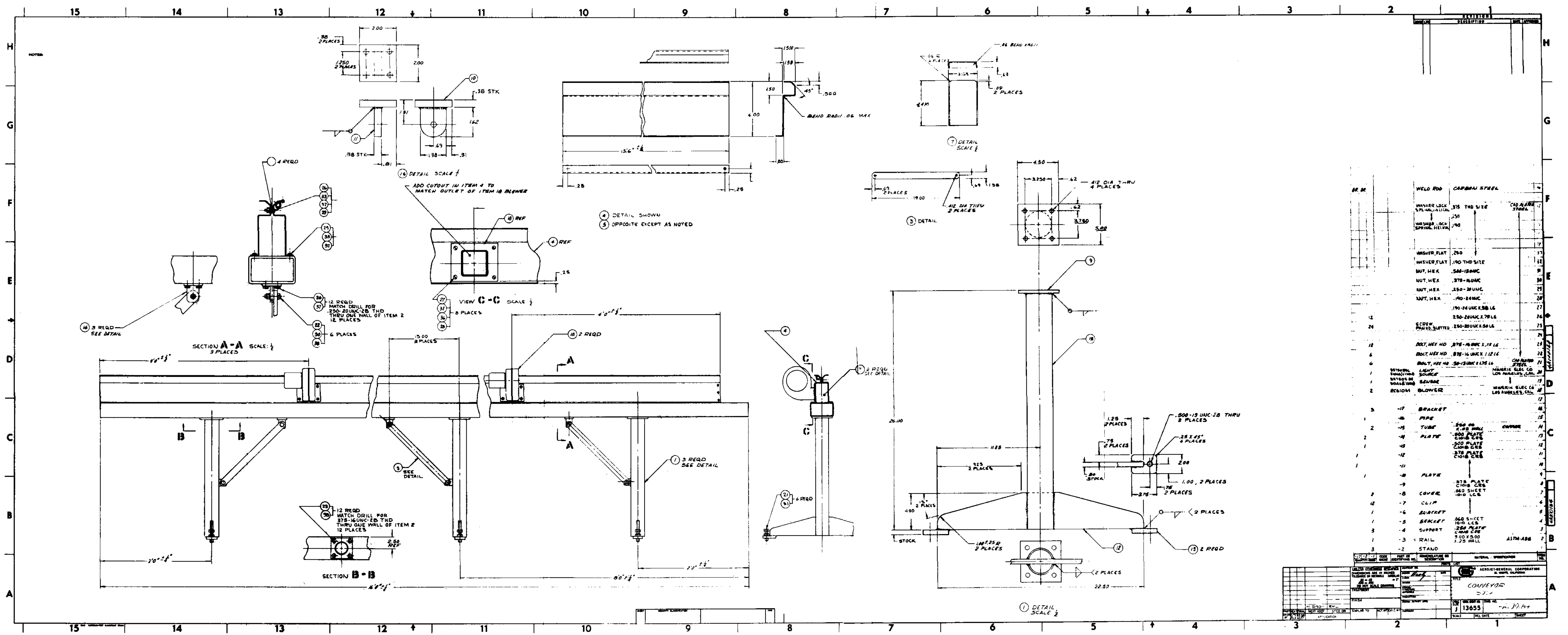
SECTION A-A SCALE: 1/2
3 PLACES
SECTION B-B
VIEW C-C SCALE 1/2
DETAIL SHOWN
OPPOSITE EXCEPT AS NOTED
ADD CUTOUT IN ITEM 4 TO MATCH OUTLET OF ITEM 18 BLOWER
BEND RADII .06 MAX
CONVEYOR
13655

Figure 12

The above experiments indicate that the lipoprotein film has been disrupted in some way, i.e., its properties have been altered during the dip-coating or drying step. It seems most likely that the film has been cracked or broken during drying and that water is necessary to maintain the integrity of the film. However, as noted above, the wet films tended to wash off the support when reintroduced into water.

Since it did not appear feasible to examine the properties of the native film by the dip-coating method, two alternative approaches were tried. One technique involved soaking the filter in a fairly concentrated solution of the coating agent. This method was used initially by Tobias and co-workers[76] to study the effects of lipids on membrane permeability. In addition to and sometimes in conjunction with the soaking procedure, the coating agent also was perfused through the porous support to achieve a maximum concentration of the agent in the support. This is possible with the lipoprotein complexes since they are usually larger than the 50 to 100 Å pores of the filters used. Cellulosic membranes are ideal for this type of study since the pores are not straight channels, but rather twisted pathways between fibers which give large surface areas for lipoprotein binding.

The following sections report permeability data on various porous supports which have been treated with lipoprotein in different ways. A number of these supports differing in composition and porosity were tested to find an optimum combination of support and treatment.

1. Apparatus

The early experiments involving measurement of membrane resistance as well as salt flux under direct osmosis conditions were carried out in a double-chambered polymethyl methacrylate direct osmosis cell. This cell was made from one large block of polymethyl methacrylate; each chamber is a cube 9 cm on a side and has a cover with a mount for a conductivity cell. The membrane is positioned between the two chambers. Leaks are prevented by two silicone rubber gaskets on either side of the membrane and four long bolts which go all the way through both chambers. The aperture over which the membrane is mounted is 2.5 cm in a diameter with an area of 4.9 $cm.^2$ Each chamber holds about 160 ml of liquid. The principal drawback with this simple cell design is that the water flux cannot be measured.

For most of the flux studies a new glass cell has been constructed, patterned after that described by Fisher and Hsiao,[77] in which water and salt fluxes can be measured simultaneously. The two chambers were fabricated from Millipore filter holder bottoms (3.8 cm frit diameter and 11.3 cm^2 frit area). This cell incorporated efficient magnetic stirring in each chamber

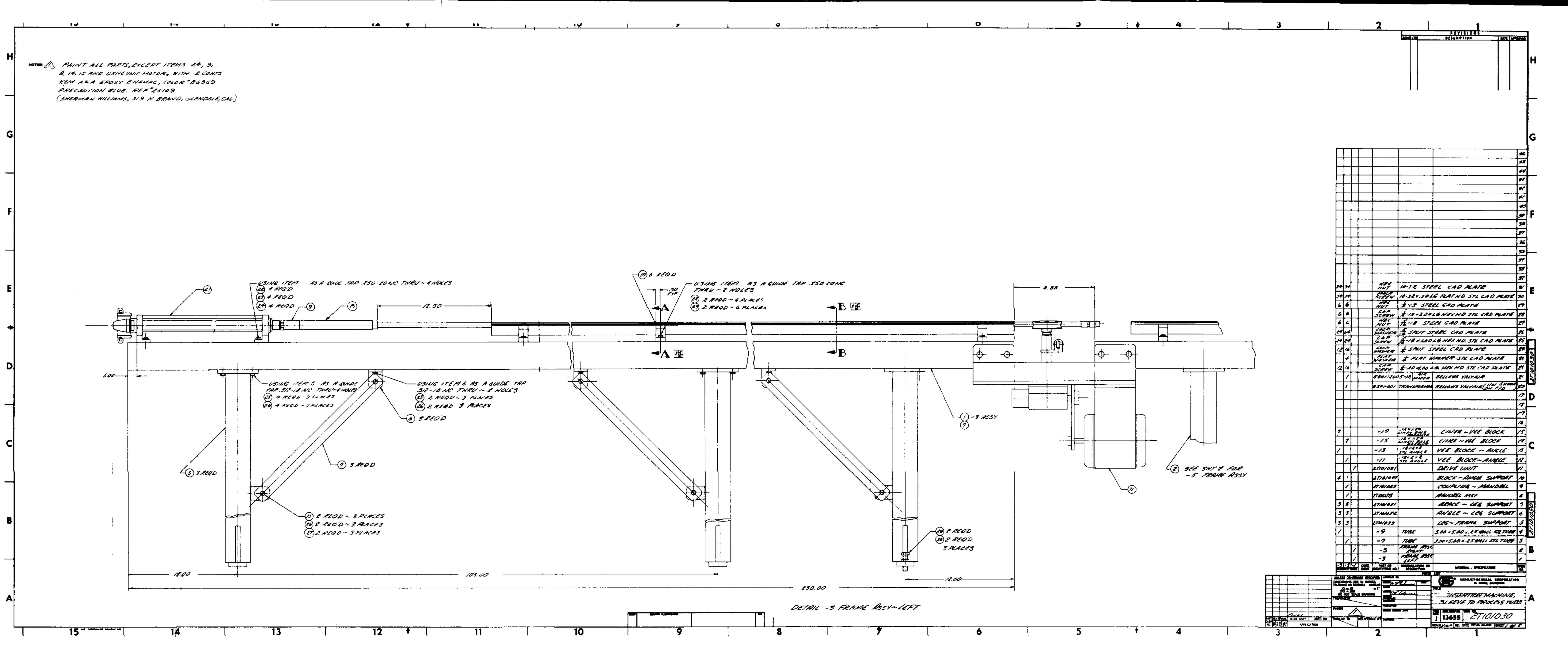
NOTES: 1. PAINT ALL PARTS, EXCEPT ITEMS 24, 9, 8, 14, 15 AND DRIVE UNIT MOTOR, WITH 2 COATS KEM A&A EPOXY ENAMAC, COLOR #B6969 PRECAUTION BLUE. REF #25109 (SHERMAN WILLIAMS, 319 N. BRAND, GLENDALE, CAL.)
DETAIL -3 FRAME ASSY~LEFT
230.00
103.00
12.00
AEROJET-GENERAL CORPORATION
INSERTION MACHINE, SLEEVE TO PROCESS TUBE
13655
2T101030

Figure 14

along with a conductivity cell (0.1 cm^{-1} cell constant) insert. Conductivity measurements were made using a General Radio Company Type 1650-A impedance bridge. The salt flux was calculated by determining how much salt had passed from one chamber to the other by the resistance change of the solution. This cell incorporated stopcocks in each half for filling, draining, and adjusting the liquid level, and ground glass joints for insertion of calibrated volumetric pipettes for measurement of volume flows. The pipettes were bent at right angles so that, when inserted in the cell, the calibrated portions of each were horizontal and at the same height to maintain zero external pressure differentail on the film during the run. The cell halves were held together by spring clamps and one rubber gasket was mounted between the membrane and the cell. The volume of each chamber was approximately 255 cm^3.

2. Millipore Filters

Millipore filters are widely used for the filtration of biological samples and are prepared from mixed esters of cellulose (presumably both acetate and nitrate esters). These filters are available with many different porosities. One of the first studies carried out was with three different Millipore filters to determine what effect pore size has on the permeability properties of the lipoprotein. The filters chosen were the Pellicon PSAC, pore size about 25 Å, Millipore VSWP, pore size 250 Å, and the Millipore PHWP, with a pore size of about 3000 Å.

In systems in which cast films were prepared from zein protein, it has been shown that crosslinking agents enhance the salt rejecting properties of the membranes.[77] Thus, in our work, three different crosslinking agents, formaldehyde, glutaraldehyde and Cymel 300 (hexamethoxymethylmelamine), were used on the filters after or during soaking. The flux of $MgSO_4$ across the coated and uncoated filters was measured in a direct osmosis experiment in which one chamber of the cell contained a 10% $MgSO_4$ solution and the other distilled water.[78] The results are given in Tables VI-VIII.

It was found that the Millipore filter with the 3000 Å pores had a salt flux which was largely unaffected by soaking in the soluble preparation or by addition of the crosslinking agents (Table VI). Presumably, the pores in this filter were so large that even when they were coated the salt could pass through them without interacting with the lipoprotein. The $MgSO_4$ flux across the 250 Å pore filter was lowered about 20-25% by the soaking and crosslinking treatment (Table VII). When these soaked filters

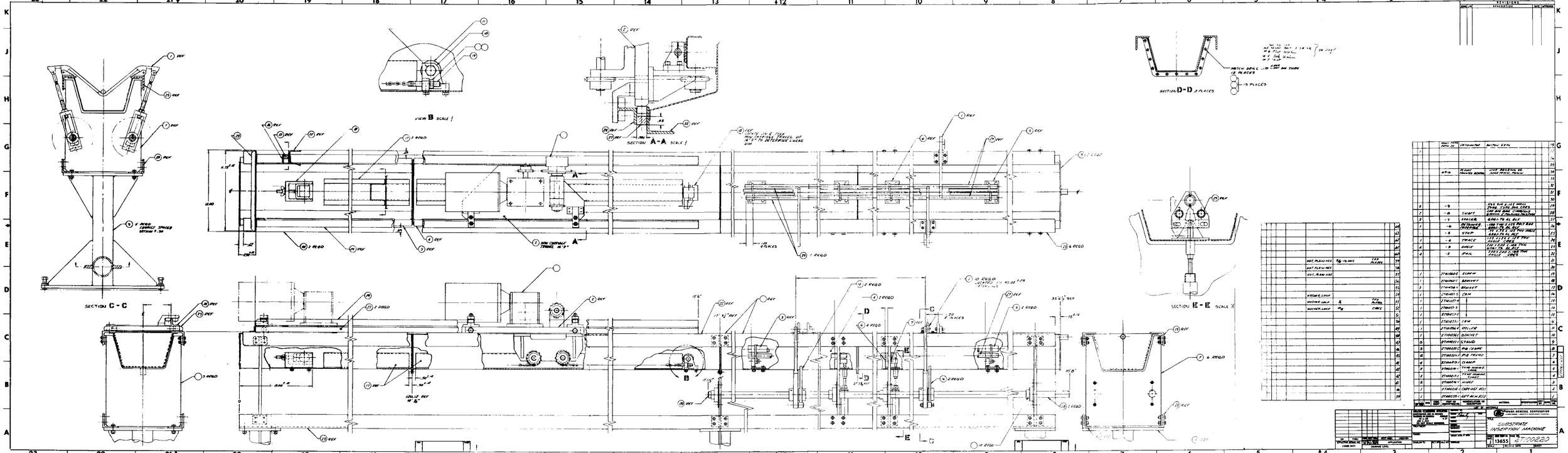
SECTION C - C
VIEW B SCALE 1
SECTION A - A SCALE 1
SECTION D-D 3 PLACES
SECTION E - E SCALE 1
SUBSTRATE INSPECTION MACHINE

Figure 16

were retested after being coated by dipping through a lipoprotein film already formed on a subphase an additional 20-25% decrease in salt flux was noted. There appeared to be a slight improvement when the film to be dip coated was first fixed with glutaraldehyde. As was mentioned previously, this latter treatment gives a somewhat stronger film. The data in Tables VI and VII were obtained using our double-chambered polymethyl methacrylate direct osmosis cell. The data in Table VIII were obtained in the improved glass direct osmosis cell, as were the data in the rest of the direct osmosis experiments. Salt and water flux measurements on six different untreated Pellicon filters were constant to within $\pm$ 5%. It was assumed that the permeabilities of the other commercial filters did not vary more than this from sheet to sheet.

The results with the Pellicon filter (Table VIII) are perhaps the most significant. Soaking the filter in the solubilized ghost preparation tends to increase the water flux and decrease the salt flux over the bare support. Comparing experiments (2) and (3), the water flux has increased by a factor of about two while the $MgSO_4$ flux has decreased by more than a factor of four. Filtration of the soluble lipoprotein preparation through the support (expts. (4) and (5) produced more erratic results. However, the glutaraldehyde treatment was omitted in these preparations and this may be a significant factor. It should be noted that the improvement in salt rejection in these systems is not obtained at the expense of the water flux and suggests that considerable increases in water flux may be possible in these membranes without sacrificing salt rejection.

Since significant differences in salt and water fluxes between the treated and untreated Millipore filters only became evident with the very tight Pellicon membranes, it was decided to limit our subsequent investigations to filters with nominal pore sizes of 100 Å or less.

3. Sartorius Filters

The salt and water fluxes through Sartorius filters (No. S 11311, cellulose nitrate, pore size approximately 100 Å) which had been treated in various ways with both intact ghost suspension and solubilized sonicate are reported in Table IX. It can be seen that with the exception of experiment (3) the presence of lipoprotein tends to decrease salt flux and increase water flux.

In this series the salt flux was diminished by up to 35X and a 20-fold increase in water flux was found. However, the

TABLE VI

$MgSO_4$ Flux Through Treated and Untreated Millipore Filters, Mean Pore Size 0.3μ (PHWP)

Treatment of Filter[a]	$MgSO_4$ Flux[b] (moles cm^{-2} sec^{-1} x 10^7)
1. Standard, water wash only	1.10
2. Soak in solubilized ghost preparation and heat dry	1.04
3. Soak in soluble preparation plus formaldehyde (1%) and heat dry	1.40
4. Soak in soluble preparation and glutaraldehyde (0.5%) and heat dry	1.21
5. Soak in soluble preparation and Cymel 300 (0.2%) and heat dry	1.11
6. Filter soluble preparation and glutaraldehyde through filter and heat dry	1.01

a. Membranes were soaked in the soluble preparation for 20 minutes, then the crosslinking agent was added and the filter soaked another 20 minutes. The coated filter was then dried overnight in an oven at 100°C.

b. The flux was constant from 1-5 hours. T = 30°C.

TABLE VII

$MgSO_4$ Flux Through Treated and Untreated Millipore Filters, Mean Pore Size 250 Å (VSWP)

Treatment of Filter[a]	$MgSO_4$ Flux[b] (moles cm^{-2} $sec^{-1} \times 10^8$)
1. Standard, water wash and heat dry	5.1
2. Soak in solubilized ghost preparation and heat dry	4.4
3. Soak in soluble preparation then Cymel 300 (0.2%) then heat dry	3.7
4. Filter soluble preparation through Millipore then soak in glutaraldehyde (0.5%) then heat dry	3.9
5. Reuse #3 and dip-coat 28 layers of film onto it	2.6
6. Reuse #4 and dip-coat 24 layers of glutaraldehyde fixed film onto it	2.3

a. The filters were soaked in the soluble preparation for 20 minutes, dried and then soaked in the crosslinking solution. They were finally dried overnight at 100°C in an oven.

b. The flux was constant from 1-5 hours. T = 30°C.

TABLE VIII

$MgSO_4$ and Water Flux Through Treated and Untreated Millipore Filters, Pore Size About 25 Å (Pellicon PSAC)

Treatment of Filter[a]	$MgSO_4$ Flux[b] (moles cm^{-2} sec^{-1} x10^9)	Water Flux (cm sec^{-1} x10^6)
1. Standard, water wash only and heat dry	9.0	29
2. Soak five times in 0.5% glutaraldehyde with heat drying after each soak	10	15
3. Soak in soluble preparation, heat dry, soak in glutaraldehyde and heat dry. This procedure was repeated five times.	2.2	33
4. Suction filter soluble preparation (1-2 ml) through membrane and heat dry.	4.7	37
5. Pressure filter ghost suspension (3 ml) through membrand and heat dry.	0.21	2.5

a. The Millipore filters were soaked for 20 minutes and dried at 100°C for 5-10 minutes between soakings.

b. The water and salt fluxes were constant from 2-5 hours. T = 25°C.

best results were not obtained with the same membrane. The marked improvement produced by glutaraldehyde treatment is again evident here in experiments (4) and (5). The glutaraldehyde seems to be particularly effective in increasing the water flux. By crosslinking the proteins in the pores of the cellulosic support, this reagent may induce conformational changes in the lipoprotein complexes, causing them to more tightly fill the pores and perhaps present a more lipid-like surface to the permeating species. It may be recalled that phospholipid bilayers are very permeable to water, but essentially impermeable to salts.[1] When experiments (3) and (7) are compared, it is apparent that filtering the sonicated ghost solution through the Sartorius membrane has a much more pronounced effect on the fluxes than soaking the membrane. This may be due to the fact that much more material is adsorbed on the membrane by the filtering technique (3-5 mg) than by the soaking technique (0.5-1 mg).

In the filtering technique, 1-2 ml of solution was added to the filter in a standard Millipore vacuum filtration apparatus. Vacuum was applied for a period of 2-4 hours by an aspirator. With the sonicated solution that period of time was usually sufficient to filter 80-90% of the solution through the membrane. With the intact ghost suspension only 20-30% of the water passed through the membrane in the same period. In all cases the membrane was not allowed to dry out on the filtration apparatus, to prevent disruption of the lipoprotein coating.

Another interesting point is that the ghost solution has much the same effect on the salt and water flux as the solubilized solution. The red cell ghosts are quite large, on the order of 4-8μ, but much smaller lipoprotein complexes are also present in solution. These complexes probably result from a small fraction of the ghost cells which were fragmented. The evidence for the smaller lipoprotein complexes being in solution along with the intact ghosts comes from the observation that when a solution of the ghost cells is cast on a $CaCl_2$ subphase a film is formed with properties quite similar to those of films cast with the sonicated solution (See Section III A). It is also possible that when the ghost cells are forced against the fibers of the filter (by vacuum filtration) they are fragmented or distorted in such a way that smaller lipoprotein complexes can penetrate deeper into the membrane. Also, the heat drying treatment would collapse the ghost cells and possibly cause some fragmentation. Thus, it can be concluded that both the ghost and sonicated solution are filling the pores of the filter in a similar way.

It does seem possible to treat the filter with too much material or possibly to pack the lipoproteins too tightly into the pores. This may be the case in experiments (4) and (8) of

TABLE IX

$MgSO_4$ and Water Flux Through Treated and Untreated Sartorius Filters (No. S11311) Under Direct Osmosis (Pore Size about 100 Å)

Treatment of Filter[a]	$MgSO_4$ Flux[b] (moles $cm^{-2} sec^{-1} \times 10^9$)	H_2O Flux (cm $sec^{-1} \times 10^6$)
1. Standard, water wash only	14	0.7
2. Water wash and heat dry - 5 times	13	<1 *
3. Soak in sonicate and heat dry - 5 times	13	0.5
4. Soak in sonicate, dip in glutaraldehyde and heat dry - 5 times	1.6	<1 *
5. Filter ghost suspension through membrane and heat dry - 2 times	5.2	4.8
6. Filter ghost suspension through membrane, dip in glutaraldehyde and heat dry - 2 times	7	14
7. Filter sonicate through membrane and heat dry - 2 times	8.3	4.8
8. Filter sonicate then ghost suspension through membrane. Dip coat 16 layers of film on the surface. Heat dry after each step	0.4	<1 *

* Water flux was variable, but at all times less than 10^{-6} cm sec^{-1}.

a. Standard procedure for soaking the membranes was to immerse them for 20 minutes in the ghost suspension (5 mg/ml) or the sonicate solution (2.5 mg/ml). If glutaraldehyde was used the wet membrane was dipped briefly into a 0.5% glutaraldehyde solution after soaking. The heat drying procedure consisted of drying the membranes in an oven at 80°C for 5-10 minutes.

b. Both the $MgSO_4$ and H_2O fluxes varied less than 10% (except where noted) over the period of the experiment, which was usually 5 hours. T = 25 ± 1°C.

Table IX, where quite large decreases in salt flux were accompanied by diminished water fluxes.

4. Other Cellulosic Filters

To complete our survey of cellulosic filters three other commercial membranes were tested briefly. These were Gelman P.E.M. cellulose triacetate (mean pore size 75 Å), Schleicher and Schuell Bac-T-Flex B-6 nitrocellulose (mean pore size 0.45μ), and Visking Dialysis Tubing made from regenerated cellulose (pore size <50 Å). These results are summarized in Table X. Surprisingly, treatment of the Gelman and S. & S. filters with either ghosts or soluble lipoprotein produced a large increase in both salt flux and osmotic water flux, quite contrary to the behavior of the Millipore and Sartorius filters. A possible explanation for this would be a large variation in the flux parameters from filter to filter in the same package or a tendency for the lipoprotein treatment to alter the porosity of the support structure. Furthermore, the heat drying was omitted in this series because these supports became brittle and fractured after drying. This could have influenced the results, since earlier dip coating work (Section IV A) showed that the drying step strengthened the bond of the lipoprotein to the cellulosic support and omitting the drying step caused the lipoprotein coats to slough off in water.

Treatment of Visking tubing with the soluble sonicate lowered the salt and water fluxes to about the same degree. This would be the expected result from fouling of the membrane by a pore plugging or surface coating process and suggests that the pores in the Visking tubing are much smaller in diameter than the lipoprotein complexes.

5. Amicon Filters

In an effort to test non-cellulosic types of porous filter supports the Amicon UM-05 ultrafiltration membrane was examined. These filters are made of mixed polyelectrolytes and have an effective pore size of less than 50 Å. The flux data from these membranes are contained in Table XI. It can be seen that here again the salt flux is decreased and the water flux is increased. Extensive measurements were not made with these filters for several reasons. One was that these membranes are much thicker than the cellulosic membranes and this caused the direct osmosis cells to leak frequently. Another problem was that heat treatment adversely affected these polyelectrolyte membranes.

TABLE X

$MgSO_4$ and Water Flux Through Treated and Untreated Gelman, Schleicher & Schuell, and Visking Filters

Filter	Treatment	$MgSO_4$ Flux (moles $cm^{-2} sec^{-1}$)	H_2O Flux ($cm\ sec^{-1}$)
1. Gelman P.E.M. (pore size 75 Å)	Standard, water wash only, test wet	$< 1.8 \times 10^{-10}$	4.9×10^{-7}
2. Gelman P.E.M	Filter 3 ml. of ghost suspension through membrane, test wet	2.3×10^{-8}	6.0×10^{-6}
3. Gelman P.E.M.	Filter 3 ml. of soluble sonicate through membrane, test wet	1.6×10^{-8}	7.2×10^{-6}
4. S.& S. Bac-T-Flex (pore size 0.45μ)	Standard, water wash only, test wet	$< 1.8 \times 10^{-10}$	9.0×10^{-7}
5. S.& S. Bac-T-Flex	Filter 3 ml. of ghost suspension through membrane, test wet	1.6×10^{-8}	2.5×10^{-6}
6. S.& S. Bac-T-Flex	Filter 3 ml. of soluble sonicate through membrane, test wet	$< 1.8 \times 10^{-10}$	1.9×10^{-6}
7. Visking Dialysis Tubing (pore size <50 Å)	Standard, water wash only, test wet	3.0×10^{-9}	7.0×10^{-6}
8. Visking Dialysis Tubing	Filter 2 ml. soluble sonicate through membrane, test wet	5.5×10^{-10}	1.5×10^{-6}

TABLE XI

$MgSO_4$ and Water Flux Through Treated and Untreated Amicon UM-05 Membranes Under Direct Osmosis, Pore Size < 50 Å

Treatment of Filter	$MgSO_4$ Flux (Moles cm^{-2} sec^{-1} x 10^9)	H_2O Flux ($cm\ sec^{-1}$ x 10^6)
1. Standard, water wash	2.1	0.03-0.8
2. Filter 2 ml of sonicate and 1 ml of ghost suspension through membrane	1.0	1.0

C. Reverse Osmosis Experiments

The results from the direct osmosis experiments were sufficiently encouraging so that similar studies were initiated to determine the effect of lipoproteins on the reverse osmosis properties of cellulosic membranes. Since the previous studies indicated that the cell membrane lipoproteins could be useful in decreasing salt flux and increasing water flux it was thought that they might function the same way when applied to asymmetric cellulose acetate membranes. Besides the usual impregnation and coating treatments, an additional technique for incorporating the lipoprotein was possible in this work. Since the cellulose acetate membranes could be prepared in the laboratory, the lipoprotein could be added to the casting solution. In this way the material should be uniformly distributed throughout the membrane.

1. Apparatus

The reverse osmosis experiments were performed on a Universal Water Corporation Reverse Osmosis Test Unit. This unit has two 2-inch diameter cells and in actual runs a standard was routinely used along with the treated membrane. The test solution used was 0.5 M NaCl at a circulation rate of 800 ml/min. and a cell pressure of 600 psi. The effluent passing through the membrane for the first half hour was discarded and samples were collected thereafter at half hour or longer intervals. The NaCl concentration of the solution passing through the membrane was determined with a Beckman sodium ion electrode in conjunction with a Beckman digital pH meter. Membrane thickness was measured on a Testing Machines, Inc., micrometer (model 549).

2. Impregnated or Coated Commercial Cellulose Acetate Membranes

In an attempt to obtain reverse osmosis results similar to those recorded from the direct osmosis work Eastman cellulose acetate membranes were impregnated with lipoprotein. The major difficulty in doing this is that these membranes cannot readily be dried after treatment, even at room temperature. Therefore, the lipoprotein preparation or the protein was filtered into the membrane with an Amicon ultrafiltration unit at 600 psi and the treated membrane was tested directly in the reverse osmosis apparatus without allowing it to become dry at any time. The results of this study are shown in Table XII. It can be seen that this treatment applied to the RO-97 membrane has little effect on either the water flux or salt rejection. This result may be due to the absence of the drying step, which is probably necessary to bind the lipoprotein or protein to the membrane matrix. Without being bound to the membrane the lipoproteins are probably being washed off the membrane by the pressurized water. Reversing the direction of filtration through the asymmetric membranes did not appreciably affect the results. Since all RO-97 membranes

were tested with the tight side against the feed, lipoprotein impregnated into the back side was probably washed out again during the RO test.

When the relatively porous HT-00 membrane was treated with ghost suspension, however, the salt rejection was raised from 0 to 30% with a corresponding sharp reduction in water flux from 232 to 1.6 gfd. When the pores of the support membrane are large enough the lipoproteins from the ghosts appear to invade them readily and set up a salt rejecting barrier. Further development of this system, e.g., by use of glutaraldehyde and special drying treatments, might produce a practical salt rejecting membrane.

A few experiments were also tried employing conventional film coating techniques to apply lipoprotein films to the surface of preformed cellulose acetate membranes. For this purpose it was first necessary to dry the cellulose acetate film. It has been shown recently[79] that when wet asymmetric cellulose acetate membranes are treated with polyvinylmethyl ether and glycerin they can be dried without losing their salt rejecting properties. Thus, the cellulose acetate membranes were cast in the usual manner, soaked overnight in the glycerin-polyvinylmethyl ether solution and then air dried. They were then taped to a sheet of plate glass and a concentrated (8-10 mg/ml) suspension of ghosts was cast over this membrane using a 5-inch doctor blade at a casting thickness of 5 mils. The ghost solution had been thickened by addition of 0.1% guar gum and 0.05% glutaraldehyde. After the first ghost film had dried a second film was cast thereon in like manner.

The results obtained from this multilayered film in the reverse osmosis tests were not encouraging (see Table XIII). Although the salt rejection did improve somewhat, the water flux was drastically reduced. The presence of the polysaccharide thickening agent in the ghost suspension might have been responsible for the low water flux observed. Attempts to cast continuous films from the soluble lipoprotein preparation or unthickened ghost suspensions onto the dried cellulose acetate film were unsuccessful.

3. Cast Cellulose Acetate Films

Table XIV contains water flux and salt rejection data for cellulose acetate membranes cast with and without lipoprotein components in the casting formulation. The general trend for the ghosts and solubilized lipoprotein was to increase the water flux, but only to a small degree compared with the direct osmosis studies. The percent salt rejection decreased a small amount in

TABLE XII

Reverse Osmosis Experiments with Impregnated Eastman Cellulose Acetate Membranes
(Pressure = 600 psi, NaCl Concentration = 0.5 M)

Membrane Treatment	Wet Thickness (mils)	Water Flux (gallons ft^{-2} day^{-1})	Salt Rejection[a] (%)
1. Eastman RO-97, untreated standard	4	8.9	71
RO-97 with 1-2 ml soluble preparation filtered through tight side of the membrane	4	10.1	76
2. Eastman RO-97, untreated standard	4	10.9	84
RO-97 with 1-2 ml soluble preparation filtered through the back side of the membrane	4	11.8	80
3. Eastman RO-97, untreated standard	4	8.9	71
RO-97 with 3 ml ghost suspension filtered through tight side of the membrane	4	6.3	70
4. Eastman RO-97, untreated standard	4	8.9	71
RO-97 with 3 ml ghost suspension filtered through back side of membrane	4	7.4	28
5. Eastman RO-97, untreated standard	4	8.7	89
RO-97 with 1-2 ml of pyridine extracted protein filtered through tight side of the membrane	4	8.9	89
6. Eastman HT-00 untreated standard	4	232	0
HT-00 with 3 ml ghost suspension filtered through membrane	4	1.6	30

[a] % Salt Rejection = $C_i - C_f / C_i \times 100$, where c_i = NaCl concentration in feed solution and C_f = NaCl concentration in the effluent solution.

TABLE XIII

Reverse Osmosis Experiments with Coated Cellulose Acetate Membranes
(Pressure = 600 psi, NaCl Conc. = 0.5 M)

Membrane Treatment	Wet Thickness (mils)	Water Flux (gallons ft^{-2} day^{-1})	Salt Rejection (%)
1. Cellulose Acetate[a], untreated standard	2.0	148	11
2. CA treated in PVM-glycerin[b]	2.0	140	4
3. Cellulose Acetate, treated in PVM-glycerin	2.0	112	4
4. CA treated in PVM-glycerin and coated twice with ghost suspension	1.0	7.85	18

a See footnote (a) of Table XIV

b PVM-glycerin solution was composed of 30% (by wt.) glycerin and 1-2% poly (vinyl methyl ether) by wt. in H_2O.[79]

both cases. Surprisingly, when the lipid and protein components were incorporated separately in the cellulose acetate film, much larger changes in water flux were noted, although the percent salt rejection was largely unaffected. The protein increased water flux while the lipid decreased it. Since lipids are hydrophobic this decrease may be due to a decreased solubility of water in the membranes containing them. The protein components, on the other hand, may be increasing the flux by increasing the solubility of water in the membrane since they are water soluble and hydrophilic.

The results of these experiments in which the lipoproteins were dissolved in the casting formulation were not as conclusive as those obtained earlier with the impregnated or coated filters (Section IV B). This may be due to the fact that in the direct osmosis studies the pores of the membrane were filled with lipoprotein, whereas in these cast films the lipoproteins are, at best, only lining the pores. Another problem with the reverse osmosis work (Table XIV) has been the low limit on lipoprotein loading, since precipitation occurs at higher concentrations. In comparing runs (1) and (2) there appears to be a trend towards increasing water flux with increasing amounts of ghost material. However, at this concentration of ghosts (0.2% W/W) a small amount of precipitate was already starting to form. Perhaps other methods of increasing the lipoprotein loading could be found. Use of other casting solvents is one possibility.

4. Cast Cellulose Acetate Butyrate Films

Parallel studies on casting cellulose acetate butyrate membranes with added lipoprotein have been attempted. It has been claimed that these membranes have even better water flux and salt rejection properties than the standard cellulose acetate films.[80]

However, in our work using the following casting formulation[80]:

	Weight %
Cellulose acetate butyrate (type EAB-171-15)	22
Triethyl phosphate	25
Glycerol	2
n-Propanol	6
Acetone	45

it was found that addition of small amounts (1-3%) of the aqueous lipoprotein solutions caused excessive thickening and sometimes precipitation. The films that were cast containing water gave widely divergent results. Films cast with a 15% cellulose acetate butyrate content were still not satisfactory, so this approach was abandoned.

TABLE XIV

Reverse Osmosis Experiments with Cast Cellulose Acetate Membranes
(Pressure = 600 psi, NaCl Concentration = 0.5 M)

Membrane Treatment[a]	Wet Thickness (mils)	Water Flux (gallons ft^{-2} day^{-1})	Salt Rejection (%)
1. Cellulose Acetate, Standard	1.3	216	14
CA and 0.1% (W/W) Ghost Suspension	1.4	244	10
2. CA, Standard	2.0	152	8
CA, 0.2% Ghost Suspension	1.0	345	14
3. CA, Standard	1.3	148	18
CA, 0.1% Solubilized Lipoprotein Solution	1.2	173	14
4. CA, Standard	1.3	142	14
CA, 0.02% Protein[b]	1.3	244	12
5. CA, Standard	1.3	173	8
CA, 0.1% Lipid[c]	2.1	33	8

a. The cellulose acetate membranes were all cast from solutions of the following composition: 15% (W/V) cellulose acetate (Eastman 398-10), 35% Formamide, 6% water and 44% acetone.[79] The lipoprotein was added directly to the CA solution before casting.

b. The protein was isolated from the rest of the cell membrane components by pyridine solubilization.

c. The lipid was isolated from the rest of the cell membrane by extraction with chloroform: isopropanol, 7:11 V/V.

V. CONCLUSIONS

Reconstituted lipoprotein membranes, although too thin to be handled and tested independently, can be incorporated into a variety of support structures, either as coatings, pore fillings, or as part of the support structure itself. Of these methods, surface coating seems to be the least effective in altering the permeability of the support member. This may arise from the hydrophilic nature of the lipoprotein films and their inability to form strong bonds to themselves or to the support matrix. Protein cross-linking agents, such as glutaraldehyde, improve the adherence of the lipoproteins to the support, but a better solution would involve use of a support which could covalently bond to the lipoprotein. Because these films are so thin, they may be unable to span a large pore in the support. This is consistent with our finding that supports with pore sizes larger than about 50 Å are little affected by treatment with membrane lipoproteins.

Application of the lipoprotein to the support by impregnating the pores followed by reconstitution within the pores has proven to be a superior method of loading supports. Even the performance of tight desalination membranes responds better to this treatment than to surface coating. The most promising systems were obtained by soaking Millipore or Sartorius filters in intact ghosts or soluble lipoprotein, drying, and then treating with glutaraldehyde. The use of glutaraldehyde seems especially effective in increasing the water flux of the membranes without sacrificing salt rejection. Filtration of the lipoprotein through the support by pressure or suction is also highly effective in filling the pores.

The evidence presented indicates clearly that the presence of lipoprotein from red cell membranes in various porous membrane supports decreases the salt flux and increases the water flux through these filters. Maximum decreases in salt flux of 97% and increases in water flux of up to 2000% have been obtained. The lipoprotein must be interacting in a specific way with the salt and water. If an inert substance were impregnated into the porous support one would expect that both the salt and water flux would decrease since the effective pore size would be diminished. It is possible that with the lipoproteins the observed decrease in salt flux is due to this effect. However, the increase in water flux cannot be explained in this manner. It seems likely that this increase is due to an increased solubility of water in the lipoprotein portion of the composite membrane.

Although relatively little was done with this approach, the properties of cellulosic membranes cast from formulations containing lipoproteins and their components have proven very interesting. In spite of the small loadings (0.02-0.2%) of natural membrane materials employed, substantial increases in water flux of CA membranes were found with intact ghosts, soluble lipoprotein and isolated protein and a sharp drop in water flux occurred with lipid alone. It may be assumed that in these composite membranes these additives may line the pores and thus influence, by their polar or nonpolar character, the water flux through the pore. Surprisingly, no corresponding effect on salt fluxes

was produced by this treatment. Further work in this area should include a major effort to increase the lipoprotein loading in these cast films.

Surface chemistry studies have shown that the reconstituted lipoproteins form tough films at an air-water interface and that the strength of the films depends on the ionic environment of the subphase. Highly complexing cations and anions form the strongest films. Cross-linking of the lipoprotein subunits in the film with glutaraldehyde also enhances film toughness. These films are much thicker than conventional protein and lipid monolayers and are believed to consist of monolayers of globular lipoprotein membrane subunits. The parallelism between the interfacial behavior of the reconstituted membrane and intact red cell ghosts is remarkable.

Further investigation of the isolated membrane components clearly supports the dominating role of the protein in these systems. The two-dimensional pressure-area characteristics of the isolated proteins are similar to those of the lipoproteins and are little affected by addition of the extracted lipid; the only large effect observed is an increase in film thickness resulting from invasion of the protein film by lipid. Closer examination of the membrane protein structure by circular dichroism revealed that little, if any, change occurs in the secondary protein structure upon solubilizing the membrane lipoproteins or even after freeing the protein of lipid. However, significant and possibly irreversible changes in the tertiary structure of the protein do appear to take place when the lipoprotein complexes are broken up. On the other hand, the reversible solubilization and reconstitution of the intact lipoprotein membrane involves no permanent change in tertiary conformation. These postulated changes in lipoprotein structure are outlined in Figure 16, in which the protein is represented by the large spheres and wiggly lines and the lipids by the small ball and stick structures. Such behavior may be rationalized by assuming that the principal forces holding together the lipoprotein complexes are lipophilic interactions between the hydrocarbon tails of the phospholipids and the nonpolar side chains of the proteins. When these complexes are destroyed, conformational changes in the protein prevent the reestablishment of these lipophilic links. Consequently, further interaction between the protein and lipid occurs through the more conventional ionic bonds between the polar heads of the lipids and the peptide units and amine and carboxylic acid residues in the protein.

To sum up, it would appear that the protein portion serves as the superstructure of the cell membrane, determining the three dimensional organization, whereas the lipid components fill in the pores of this superstructure in order to control and select molecules that may penetrate. Dramatic changes in cell membrane permeability, such as those that occur in nerve impulse transmission, are probably caused by allosteric changes in the enzymes contained in the protein superstructure.

VI. RECOMMENDATIONS

Much further work is necessary to develop practical synthetic models

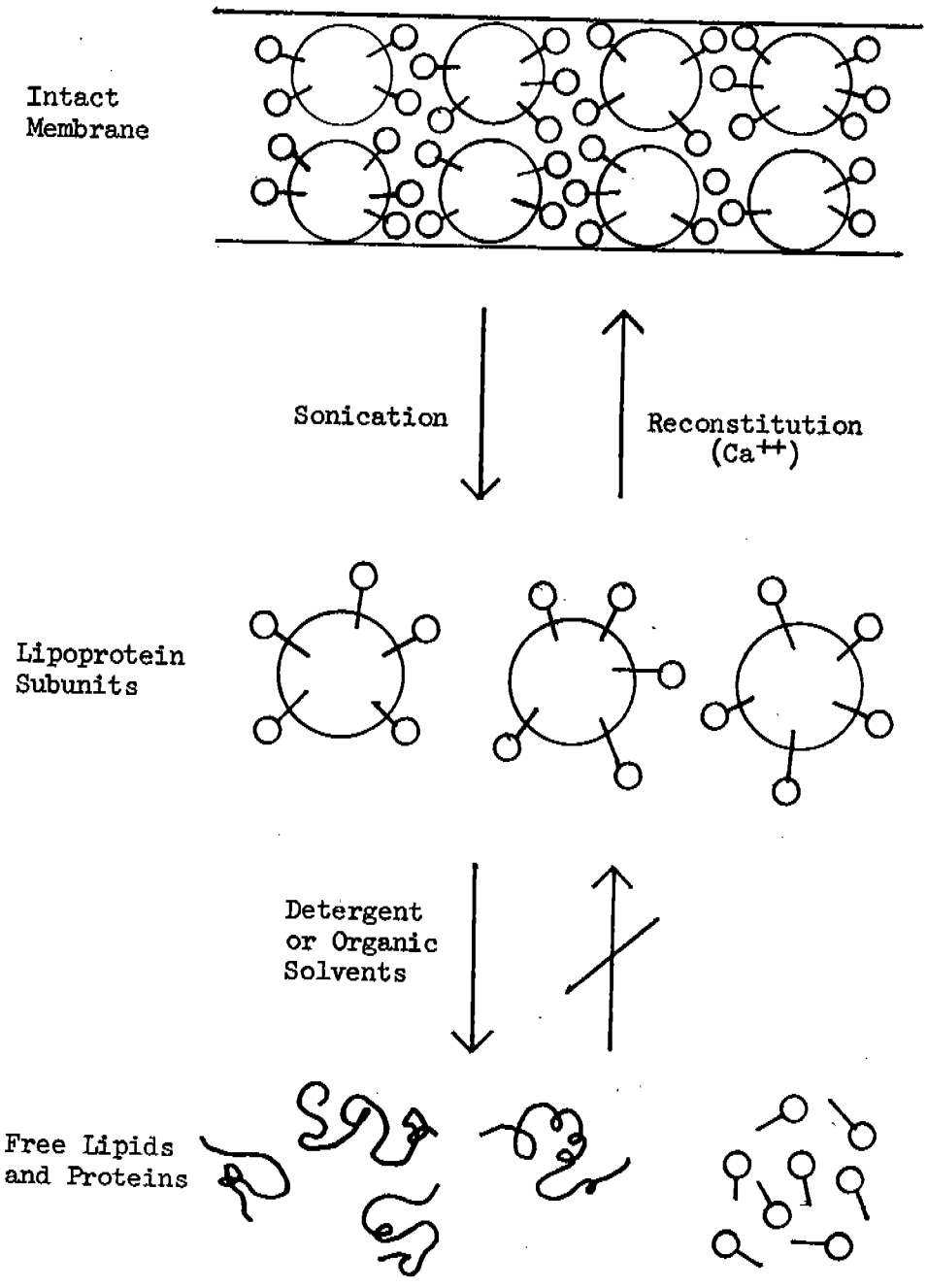

Figure 16 - Postulated changes in lipoprotein organization

of natural membranes which can compete with currently available desalination membranes. Based on the results of this exploratory study, promising leads have been found which warrent further work. Several are listed below.

1. Effect of lipoproteins on increased water flux without loss of salt barrier. Much more work is needed on both impregnated and composite cast membrane systems containing lipoproteins and their components in order to optimize this effect and understand the principles involved.

2. Improve lipoprotein loading of cast CA membranes. New formulations are needed in which larger amounts of lipoporteins can be loaded into cast CA membranes. Many types of both synthetic and natural lipid and protein substances should be formulated into cast cellulose acetate membranes.

3. Development of porous support materials having high affinity for lipoproteins. New types of synthetic support membranes should be sought to which lipoproteins may be covalently bonded or tightly complexed.

4. Determine the uniqueness of red cell membrane lipoproteins in modifying flux characteristics of supports. Repeat permeability studies with other lipoproteins, e.g., low and high density serum lipoproteins, chylomicrons, bacterial, chloroplast and mitochondrial lipoproteins.

5. Determine long term effects of lipoprotein treatment on the flux characteristics of CA membranes. Do lipoproteins or their components alter the tendency of cellulosic membranes to foul in the presence of proteinaceous feeds?

6. Active transport of salt. Since Na,K-ATPase is known to survive solubilization of the red cell membrane, the lipoprotein loaded support systems developed in this project should be good models in which to demonstrate active transport in a reconstituted membrane system.

7. Development of non-biodegradable analogs of natural lipoproteins for long term use in desalination. Are their properties comparable to those of natural membranes?

8. Further study of lipid-protein interaction in biological membranes. Much more effort is necessary to identify the subtle effects of the lipid and protein components on the permeability properties of biological membranes.

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